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ABSTRACT

Maytenus buchananii is worldwide distributed small evergreen tree traditionally used as anti-tumor, anti-ulcer, anti-inflammatory, and as antimicrobial. The column chromatography of the MeOH extract of *M. buchananii* leaves followed by purification of different fractions led to the isolation of nine known flavonoids. Their structures were elucidated on the basis of spectra analysis, and by comparison with those from the literature. The antibacterial activity was assessed by performing minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and the time-kill kinetic study while the antioxidant activity was determined by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) and trolox equivalent antioxidant capacity (TEAC) assays. All of the extracts/compounds showed antibacterial activity against the strains of Gram-positive bacteria, *Staphylococcus aureus*, and Gram-negative multi-drug resistance bacteria, *Vibrio cholerae* (causative agent of cholera) and *Shigella flexneri* (causative agent of shigellosis). Compounds **2** (MIC = 32 to 128 μg/mL) and **6** (MIC = 16 to 64 μg/mL) showed the largest antibacterial activities with the best MIC (16 μg/mL) recorded with compound **6** on *Staphylococcus aureus*. The results of both DPPH

and TEAC assays found compounds **3** (EC_{50} = 1.38 µg/mL; TEAC= 89.69 µg/mL), **5** (EC_{50} = 1.56 µg/mL; TEAC= 90.93 µg/mL) and **6** (EC_{50} = 1.42 µg/mL; TEAC= 89.76 µg/mL) to exhibit the most antioxidant activity. Results obtained from this study may help to exploit the use of the *M. buchananii* leaf extracts and some of their flavonoid compounds as pharmacological ingredients for promoting health, especially for cholera/shigellosis and chronic diseases associated with oxidative stress.

Keywords: *Maytenus buchananii*; Celastraceae; leaf extract; phytochemical analysis; flavonoids; antioxidant; antimicrobial; time-kill study.

1. Introduction

Acute watery diarrhea accounts for 80% of the cases (death account for 50%) in the developing world (Tullock and Richards, 1993). Among the diarrheal diseases, cholera is a serious epidemic disease caused by the gram-negative bacterium *Vibrio cholerae* (Nair et al., 1994). *Vibrio cholerae*, serotypes O1 and O139 has ability to produce an enterotoxin, cholera toxin that is a major determinant of virulence for cholera. Among the other virulence factors, ElTor hemolysin produced by *Vibrio cholerae* is also reportedly a potent toxin with both enterotoxic and cytotoxic activities (Ichinose et al., 1987; Ramamurthy et al., 1993). Some drugs such as racecadotril and loperamide used to treat the secretary diarrhea, have side effects such as bronchopasm, vomiting and fever, and loperamide should not be administrated to children below 6 years of age, patients with constipation, and intestinal obstruction (Roge et al., 1993; Salazar et al., 2000). Emergence of multiply drug-resistant *Vibrio cholerae* is a serious clinical problem in the treatment and containment of the disease, as reflected by the increase in the fatality rate from 1% to 5.3% after the emergence of drug-resistant strains in Guinea-Bissau during the 1996–1997 epidemic of cholera (Dalsgaard et al., 1999). The

increasing incidence of drug-resistant pathogens has drawn the attention of the pharmaceutical and scientific communities towards studies on the potential antimicrobial activity of plant-derived products, an untapped source of antimicrobial chemotypes, which are used in traditional medicine in different countries. *Maytenus buchananii* (Loes.) R. Wilczek belonging to the Celastraceae family is wordwide distributed small evergreen tree 2-12 m high, with paired spinesup to 2.8 mm long. It has been reported that plants of this genus are widely used in folk medicine as anti-tumor, anti-asthmatic, anti-ulcer, treatment of stomach problems, anti-inflammatory, analgesic and as antimicrobial (Ghazanfar, 1994; Muhammad et al., 2000; Orabi et al., 2001; Al Haidari et al., 2002). The biological activities associated to *Maytenus* species have been assigned to different classes of secondary metabolites such as triterpenes (Sannomiya et al., 1998; El Tahir et al., 2001; Lindsey et al., 2006; Martucciello et al., 2010), phenolics glucosides (Da Silva et al., 2008), alkaloids (Corsino et al., 1998a; Orabi et al., 2001) and flavonoids (Vilegas et al., 1999; Lindsey et al., 2006; Dias et al., 2007). Flavonoids, previously called bioflavonoids and included in aromatic compounds, are important constituents of plants. The basic structural feature of flavonoid compounds is the 2-phenylbenzopyrane or flavane nucleus, consisting of two benzene rings linked through a heterocyclic pyrane ring. In total, there are 14 classes of flavonoids, differentiated on the basis of the chemical nature and position of substituents on the different rings. For centuries, preparations containing these compounds as the principal physiologically active constituents have been used to treat human diseases such as infections associated with bacteria and those related to oxidative stress. Many investigations revealed that flavonoids content contribute to the antimicrobial (Araujo et al., 2011; Garcia et al., 2012; Djouossi et al., 2015) and antioxidant (Pietta et al., 1998; Djouossi et al., 2015) activities of plants. In the course of our continuing search for secondary metabolites of biological importance from Cameroonian

medicinal plants, we evaluate the antibacterial and antioxidant activities of extracts and flavonoids from the leaves of *M. buchananii*.

2. Materials and methods

2.1. General

Optical rotations were measured on a Perkin-Elmer 341 polarimeter. ^{1}H and ^{13}C-NMR spectra were recorded on a Bruker Avance III 600 spectrometer equipped with a cryoplatform (^{1}H at 600 MHz and ^{13}C at 150 MHz). 2D-NMR experiments were performed using standard Bruker microprograms (Xwin-NMR version 2.1 software). Chemical shifts (δ) are reported in parts per million (ppm) with the solvent signals as reference relative to TMS (δ=0) as internal standard, while the coupling constants (*J* values) are given in Hertz (Hz). The IR spectra were recorded with a Shimadzu FT-IR-8400S spectrophotometer. UV spectra were determined as methanol solution with a Cary 50 UV/VIS Spectrophotometer. HR-TOFESIMS experiments were performed using a Micromass Q-TOF micro instrument (Manchester, UK) with an electrospray source. The samples were introduced by direct infusion in a solution of MeOH at a rate of 5 µL/min. Column chromatography (CC) was performed on silica gel 60 (70-230 mesh, Merck) and gel permeation on Sephadex LH-20 while TLC was carried out on silica gel GF_{254} pre-coated plates with detection accomplished by spraying with 50% H_2SO_4 followed by heating at 100 °C, or by visualizing with an UV lamp at 254 and 365 nm.

2.2. Plant material

The leaves of *Maytenus buchananii* was collected at Dschang, Menoua Division, West Region of Cameroon, in March 2012. Authentication was done by Victor Nana, a botanist of the Cameroon National Herbarium, Yaoundé, where a voucher specimen (N^{o} 12659/SFR/CAM) is deposited.

2.3. Extraction and isolation

The dried and powdered plant material (4 Kg) was extracted two times (each for 24 h) with 15 L MeOH at room temperature. The filtrate obtained was concentrated under reduced pressure to yield a dark residue (540 g). This crude extract was fractionated with hexane, EtOAc and *n*-BuOH, yielding after evaporation to dryness 58, 141 and 65 g of hexane, EtOAc and *n*-BuOH fractions, respectively and 235 g of a brown gum.

A portion (50 g) of the hexane fraction was subjected to silica gel column chromatography (CC) eluting with gradient mixtures of hexane-EtOAc (1:0; 9:1; 4:1; 7:3; 1:1; 3:7; and 0:1). Fractions of 250 mL were collected and combined on the basis of their TLC profiles to give four fractions noted FH1 to FH4. Fraction FH4 was purified by column chromatography over silica gel with hexane-EtOAc (1:4) and fractions (25 mL each) were collected, yielding five sub-fractions. Purification of sub-fraction [10-23] on silica gel column chromatography with hexane-EtOAc (1:4) led to the isolation of compound **1** (13 mg).

130 g of the EtOAc fraction was submitted to silica gel column chromatography eluted with gradient mixtures of hexane-EtOAc (1:1; 3:7; and 0:1) followed by EtOAc-MeOH (9:1; 4:1; and 3:2). Fractions of 250 mL were collected and combined on the basis of their TLC profiles to give six fractions noted FE1 to FE6. Fraction FE2 was purified by column chromatography over silica gel with hexane-EtOAc (1:1) and fractions (50 mL each) were collected to give three sub-fractions. Sub-fraction [13-21] was subjected to Sephadex LH-20 column chromatography with MeOH to give compound **3** (12 mg). Fraction FE3 was also purified by column chromatography over silica gel with hexane-EtOAc (3:7) and fractions (50 mL each) were collected to give six sub-fractions. Purification of sub-fractions [15-25] and [36-51] afforded compounds **2** (32 mg) and **4** (36 mg) respectively. Fraction FE4 was repeatedly purified by column chromatography over silica gel with hexane-EtOAc (1:4) and

fractions (75 mL each) were collected, yielding six sub-fractions. Purification of sub-fractions [14-24] on silica gel with hexane-EtOAc (2:3) gave compound **7** (12 mg), while sub-fractions [41-50] and [106-121] were passed through Sephadex LH-20 column chromatography using MeOH and purified on silica gel column chromatography with hexane-EtOAc (3:7) and (1:9) respectively to afford compounds **5** (14 mg) and **6** (24 mg) respectively.

50 g of the gum were repeatedly subjected to Sephadex LH-20 column chromatography using MeOH to separate compounds (Extract) from the trail (Rest). This extract (35 g) was purified by column chromatography over silica gel with EtOAc. Fractions of 100 mL were collected and combined on the basis of their TLC profiles to give six fractions noted FG1 to FG6. Fraction FG1 was purified on silica gel column chromatography eluted with hexane-EtOAc (4:1) and fractions (25 mL each) were collected to give four sub-fractions. Purification of sub-fraction [5] gave compound **8** (17 mg). Fraction F3G was purified over silica gel column chromatography eluted with the mixture EtOAc-MeOH-H_2O (95-3-2) to give compound **9** (20 mg).

2.4. Structural identification of the isolated compounds

Samples for NMR experiments were dissolved in CD_3OD on a BRUKER Avance DRX 600 Spectrometer (600MHz for ^{1}H and 150MHz for ^{13}C). Column chromatography was performed on silica gel 60 (70-230 mesh, Merck) and sephadex LH-20. Fractions were monitored by TLC using Merck pre-coated silica gel sheets (60 F_{254}), and spots were visualized under UV light (254 and 365 nm) and by spraying with 50% H_2SO_4 and heating at 100 °C. 1D and 2D-NMR experiments (COSY, TOCSY, ROESY, HSQC-*Jmod*, and HMBC) were performed using standard Bruker pulse programs (XW in NMR version 2.1).

Quercetin-3-*O*-*α*-L-arabinopyranoside **1**: yellow powder. UV: 250, 279, 260, 375 nm. IR ν_{max} (KBr, cm^{-1}): 3289, 2995, 1660, 1505. ^{13}C-NMR (CD_3OD, 150MHz) *δ:* 157.3 (C-2),

134.2 (C-3), 178.4 (C-4), 161.7 (C-5), 98.5 (C-6), 164.6 (C-7), 93.3 (C-8), 157.0 (C-9), 104.2 (C-10), 121.4 (C-1'), 116.0 (C-2'), 144.6 (C-3'), 148.6 (C-4'), 114.8 (C-5'), 121.6 (C-6') for aglycone; 103.2 (C-1"), 71.5 (C-2"), 72.8 (C-3"), 67.7 (C-4"), 65.6 (C-5") for sugar moiety. ^{1}H-NMR (CD_3OD, 600MHz) *δ:* 6.23 (1H, d, *J* = 2.0 Hz, H-6), 6.42 (1H, d, *J* = 2.0 Hz, H-8), 7.77 (1H, d, *J* = 2.2 Hz, H-2'), 6.89 (1H, d, *J* = 8.5 Hz, H-5'), 7.60 (1H, dd, *J* = 2.2 and 8.5 Hz, H-6') for aglycone; 5.18 (1H, d, *J* = 6.6 Hz, H-1"), 3.92 (1H, dd, *J* = 6.6 and 8.4 Hz, H-2"), 3.67 (1H, dd, *J* = 3.1 and 8.4 Hz, H-3"), 3.83 (1H, m, H-4"), 3.47 (1H, m, H-5"a), 3.86 (1H, m, H-5"b) for sugar moiety.

Epicatechin **2**: yellow powder. UV: 282 and 219 nm. IR ν_{max} (KBr, cm^{-1}): 2600-3400 (broad), 1620, 1520, 1470, 1380, 1280, 1240. ^{13}C-NMR (CD_3OD, 150 MHz) *δ:* 78.5 (C-2), 66.1 (C-3), 27.9 (C-4), 156.6 (C-5), 95.0 (C-6), 156.3 (C-7), 94.5 (C-8), 156.0 (C-9), 98.7 (C-10), 130.9 (C-1'), 113.9 (C-2'), 144.4 (C-3'), 144.6 (C-4'), 114.5 (C-5'), 118.0 (C-6'). ^{1}H-NMR (CD_3OD, 600 MHz) *δ:* 4.84 (1H, m, H-2), 4.19 (1H, m, H-3), 2.76 (1H, dd, *J* = 2.9 and 16.7 Hz, H-4a), 2.88 (1H, dd; *J* = 4.6 and 16.7 Hz, H-4b), 5.96 (1H, d, *J* = 2.3 Hz, H-6), 5.93 (1H, d, *J* = 2.3 Hz, H-8), 6.99 (1H, d, *J* = 1.8 Hz, H-2'), 6.78 (1H, d, *J* = 8.1 Hz, H-5'), 6.82 (1H, dd, *J* = 1.8 and 8.1 Hz, H-6').

Quercetin **3**: green powder. UV: 253, 278, 261, 370 nm. IR ν_{max} (KBr, cm^{-1}): 1660, 1594, 1506, 1353, 1205. ^{13}C-NMR (CD_3OD, 150 MHz) *δ:*156.8 (C-2), 135.8 (C-3), 175.9 (C-4), 161.1 (C-5), 97.8 (C-6), 164.2 (C-7), 93.0 (C-8), 147.4 (C-9), 103.1 (C-10), 122.7 (C-1'), 114.8 (C-2'), 144.8 (C-3'), 146.6 (C-4'), 114.6 (C-5'), 120.2 (C-6'). ^{1}H-NMR (CD_3OD, 600 MHz) *δ:* 6.21 (1H, d, *J* = 2.0 Hz, H-6), 6.41 (1H, d, *J* = 2.0 Hz, H-8), 7.76 (1H, d, *J* = 2.2 Hz, H-2'), 6.91 (1H, d, *J* = 8.5 Hz, H-5'), 7.66 (1H, dd, *J* = 2.2 and 8.5 Hz, H-6').

Kaempferol-3-*O*-*α*-L-rhamnopyranoside **4**: yellow powder. UV: 255, 267, 372 nm. IR ν_{max} (KBr, cm^{-1}): 3290, 2981, 1663, 1592, 1457, 1351, 1210. ^{13}C-NMR (CD_3OD, 150 MHz), *δ:* 157.9 (C-2), 134.8 (C-3), 178.2 (C-4), 161.8 (C-5), 98.4 (C-6), 164.5 (C-7), 93.4 (C-8),

157.1 (C-9), 104.5 (C-10), 121.2 (C-1'), 130.5 (C-2'), 115.1 (C-3'), 160.2 (C-4'), 115.1 (C-5'), 130.5 (C-6') for aglycone; 102.1 (C-1"), 70.5 (C-2"), 70.7 (C-3"), 71.8 (C-4"), 70.6 (C-5"), 16.3 (C-6") for sugar moiety. ^{1}H-NMR (CD_3OD, 600 MHz) *δ:*6.22 (1H, d, *J* = 2.1 Hz, H-6), 6.39 (1H, d, *J* = 2.1 Hz, H-8), 7.78 (2H, d, *J* = 6.8 Hz, H-2' and H-6'), 6.95 (2H, d, *J* = 6.8 Hz, H-3' and H-5') for aglycone; 5.40 (1H, d, *J* = 1.6 Hz, H-1"), 4.24 (1H, m, H-2"), 3.73 (1H, m, H-3"), 3.35 (1H, m, H-4"), 3.36 (1H, m, H-5"), 0.98 (3H, m, H-6") for sugar moiety.

Quercetin-3-*O*-*α*-L-rhamnopyranoside **5**: yellow powder. UV: 257, 266, 274, 374 nm. IR ν_{max} (KBr, cm^{-1}): 3288, 2990, 1597, 1500, 1459, 1355, 1094. ^{13}C-NMR (CD_3OD, 150 MHz) *δ:*157.9 (C-2), 134.8 (C-3), 178.2 (C-4), 161.8 (C-5), 98.4 (C-6), 164.5 (C-7), 93.3 (C-8), 157.1 (C-9), 104.5 (C-10), 121.4 (C-1'), 116.5 (C-2'), 145.0 (C-3'), 148.4 (C-4'), 114.7 (C-5'), 121.6 (C-6') for aglycone; 102.1 (C-1"), 70.5 (C-2"), 70.7 (C-3"), 71.8 (C-4"), 70.6 (C-5"), 16.3 (C-6") for sugar moiety. ^{1}H-NMR (CD_3OD, 600 MHz) *δ:* 6.23 (1H, d, *J* = 2.1 Hz, H-6), 6.39 (1H, d, *J* = 2.1 Hz, H-8), 7.36 (1H, d, *J* = 2.1 Hz, H-2'), 6.93 (1H, d, *J* = 8.3 Hz, H-5'), 7.33 (1H, dd, *J* = 2.1 and 8.3 Hz, H-6') for aglycone; 5.37 (1H, d, *J* = 1.1 Hz, H-1"), 4.24 (1H, m, H-2"), 3.77 (1H, m, H-3"), 3.37 (1H, m, H-4"), 3.43 (1H, m, H-5"), 0.96 (3H, d, *J* = 6.2 Hz, H-6") for sugar moiety.

Quercetin-3-*O*-*β*-D-glucopyranoside **6**: yellow powder. UV: 257, 263, 325, 429 nm. IR ν_{max} (KBr, cm^{-1}): 3286, 2993, 1660, 1597, 1455, 1350. ^{13}C-NMR (CD_3OD, 150 MHz) *δ:* 156.9 (C-2), 134.3 (C-3), 178.0 (C-4), 161.2 (C-5), 98.6 (C-6), 164.6 (C-7), 93.4 (C-8), 156.7 (C-9), 104.3 (C-10), 121.4 (C-1'), 116.4 (C-2'), 144.8 (C-3'), 148.4 (C-4'), 114.9 (C-5'), 121.6 (C-6') for aglycone; 103.6 (C-1"), 71.7 (C-2"), 73.7 (C-3"), 68.6 (C-4"), 75.9 (C-5"), 60.3 (C-6") for sugar moiety. ^{1}H-NMR (CD_3OD, 600 MHz)*δ:* 6.25 (1H, d, *J* = 2.0 Hz, H-6), 6.46 (1H, d, *J* = 2.0 Hz, H-8), 7.86 (1H, d, *J* = 2.3 Hz, H-2'), 6.91 (1H, d, *J* = 8.4 Hz, H-5'), 7.64 (1H, dd, *J* = 2.4 and 8.4 Hz, H-6') for aglycone; 5.30 (1H, d, *J* = 7.8 Hz, H-1"), 3.82

(1H, m, H-2”), 3.58 (1H, m, H-3”), 3.87 (1H, m, H-4”), 3.51 (1H, m, H-5”), 3.56 (1H, m, H-6”a), 3.67 (1H, m, H-6”b) for moiety sugar.

Epigallocatechin **7**: yellow powder. UV: 282 and 219 nm. IR ν_{max} (KBr, cm^{-1}): 2600-3400 (broad), 1620, 1520, 1470, 1380, 1280, 1240. ^{13}C-NMR (CD_3OD, 150 MHz) *δ:* 78.5 (C-2), 66.1 (C-3), 27.7 (C-4), 156.6 (C-5), 95.0 (C-6), 156.3 (C-7), 94.4 (C-8), 156.0 (C-9), 98.7 (C-10), 130.1 (C-1’), 105.6 (C-2’), 145.3 (C-3’), 132.2 (C-4’), 145.3 (C-5’), 105.6 (C-6’). ^{1}H-NMR (CD_3OD, 600 MHz), *δ:* 4.77 (1H, m, H-2), 4.19 (1H, m, H-3), 2.75 (1H, dd, *J* = 2.9 and 16.6 Hz, H-4a), 2.87 (1H, dd, *J* = 4.6 and 16.6 Hz, H-4b), 5.96 (1H, d, *J* = 2.3 Hz, H-6), 5.93 (1H, d, *J* = 2.3 Hz, H-8), 6.53 (2H, s, H-2’ and H-6’).

Kaempferol **8**: yellow powder. UV: 254, 260, 280, 372 nm. IR ν_{max} (KBr, cm^{-1}): 2992, 1658, 1592, 1455, 1350, 1208, 1094. ^{13}C-NMR (CD_3OD, 150 MHz) *δ:* 158.3 (C-2), 137.2 (C-3), 177.4 (C-4), 162.5 (C-5), 99.3 (C-6), 165.8 (C-7), 94.5 (C-8), 160.6 (C-9), 104.6 (C-10), 123.8 (C-1’), 130.7 (C-2’), 116.3 (C-3’), 148.1 (C-4’), 116.3 (C-5’), 130.7 (C-6’). ^{1}H-NMR (CD_3OD, 600 MHz) *δ:* 6.26 (1H, d, *J* = 2.0 Hz, H-6), 6.45 (1H, d, *J* = 2.0 Hz, H-8), 8.12 (2H, d, *J* = 6.9 Hz, H-2’ and H-6’), 6.94 (2H, d, *J*= 6.9 Hz, H-3’ and H-5’).

Quercetin-3-*O*-[*α*-L-rhamnopyranosyl-(1→6)-*β*-D-glucopyranoside] **9**: yellow powder. UV: 254, 268, 310, 355 nm. IR ν_{max} (KBr, cm^{-1}): 3288, 2987, 1598, 1504, 1460, 1348, 1209, 1092. ^{13}C-NMR (CD_3OD, 150 MHz) *δ:*157.5 (C-2), 134.4 (C-3), 178.0 (C-4), 161.6 (C-5), 98.6 (C-6), 165.0 (C-7), 93.5 (C-8), 157.1 (C-9), 104.1 (C-10), 121.4 (C-1’), 116.5 (C-2’), 144.4 (C-3’), 148.6 (C-4’), 114.7 (C-5’), 121.6 (C-6’) for aglycone; 104.6 (C-1”), 71.7 (C-2”), 73.7 (C-3”), 68.8 (C-4”), 73.9 (C-5”), 65.9 (C-6”) for glucosyl sugar, 100.5 (C-1”’), 70.7 (C-2”’), 70.9 (C-3”’), 72.5 (C-4”’), 68.3 (C-5”’), 16.6 (C-6”’) for rhamnosyl sugar. ^{1}H-NMR (CD_3OD, 600 MHz) *δ:* 6.24 (1H, d, *J* = 2.0 Hz, H-6), 6.44 (1H, d, *J* = 2.0 Hz, H-8), 7.88 (1H, d, *J* = 2.2 Hz, H-2’), 6.88 (1H, d, *J* = 8.5 Hz, H-5’), 7.63 (1H, dd, *J* = 2.2 and 8.5 Hz, H-6’) for aglycone; 5.10 (1H, d, *J* = 7.8 Hz, H-1”), 3.86 (1H, m, H-2”), 3.58 (1H, m, H-3”), 3.83

(1H, m, H-4”), 3.67 (1H, m, H-5”), 3.43 (1H, m, H-6”a), 3.77 (1H, m, H-6”b) for glucosyl sugar, 4.54 (1H, d, *J* = 1.4 Hz, H-1”’), 3.60 (1H, m, H-2”’), 3.52 (1H, m, H-3”’), 3.31 (1H, m, H-4”’), 3.55 (1H, m, H-5”’), 0.95 (3H, m, H-6”’) for rhamnosyl sugar.

2.5. Antimicrobial assay

2.5.1. Microorganisms

A total of six bacterial strains were tested for their susceptibility to compounds and these strains were taken from our laboratory collection (kindly provided by Dr. T. Ramamurthy, NICED, Kolkata). Among the clinical strains of *Vibrio cholerae* used in this study, strains NB2 and SG24(1) belonged to O1 and O139 serotypes, respectively. These strains were able to produce cholera toxin and hemolysin (Bag et al., 2008; Thakurta et al., 2007). The other strains used in this study were *V. cholerae* non-O1, non-O139 (strains CO6 and PC2) (Bag et al., 2008); and *Shigella flexneri* (Acharyya et al., 2015). The *V. cholerae* non-O1 and non-O139 strains, were positive for hemolysin production but negative for cholera toxin production (Bag et al., 2008). The American Type Culture Collection (ATCC) strain, *Staphylococcus aureus* ATCC 25923, was used for quality control. The bacterial strains were maintained on agar slant at 4 °C and subcultured on a fresh appropriate agar plates 24 h prior to any antibacterial test. The Mueller Hinton Agar (MHA) was used for the activation of bacteria. The Mueller Hinton Broth (MHB) and nutrient agar (Hi-Media) were used for the MIC and MBC determinations respectively.

2.5.2. Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

MIC values were determined by a broth micro-dilution method as described earlier (Nyaa et al., 2009) with slight modifications. Each test sample was dissolved in

dimethylsulfoxide (DMSO) and the solution was then added to Mueller Hinton Broth (MHB) for bacteria to give a final concentration of 1024 μg/mL. This was serially diluted twofold to obtain a concentration range of 0.50–1024 μg/mL. Then, 100 μL of each concentration was added in each well (96-well microplate) containing 95 μL of MHB and 5 μL of inoculum for final concentrations varying from 0.25–512 μg/mL. The inoculum was standardized at 1.5 $\times 10^6$ CFU/mL by adjusting the optical density to 0.1 at 600 nm using a JENWAY 6105 UV/Vis spectrophotometer. The final concentration of DMSO in each well was < 1% [preliminary analyses with 1% (v/v) DMSO did not inhibit the growth of the test organisms]. The negative control well consisted of 195 μL of MHB and 5 μL of the standard inoculum. The plates were covered with sterile lids, then agitated to mix the contents of the wells using a plate shaker and incubated at 35 °C for 24 h. The assay was repeated three times. The MIC values of samples were determined by adding 50 μL of a 0.2 mg/mL *p*-iodonitrotetrazolium violet solution followed by incubation at 35 °C for 30 min. Viable microorganisms reduced the yellow dye to a pink color. MIC values were defined as the lowest sample concentrations that prevented this change in color indicating a complete inhibition of microbial growth. For the determination of MBC values, a portion of liquid (5 μL) from each well that showed no growth of microorganism was plated on Mueller Hinton Agar and incubated at 35 °C for 24 h. The lowest concentrations that yielded no growth after this subculturing were taken as the MBC values (Tamokou et al., 2011). Ciprofloxacin and ampicillin (Sigma-Aldrich, Steinheim, Germany) were used as positive controls.

2.5.3. The time-kill kinetic study (for antimicrobial drugs) against Vibrio cholerae SG24 (1)

Time-kill dynamic assay was performed using broth microdilution method as previously described (Avila et al., 1999) with minor modifications. Cultures of bacteria in MHB (1×10^6 CFU/mL) were incubated separately at 37 °C for 0, 2, 4, 6, 10, and 24 hours in

the absence (control) and in the presence of the drug/extract at MIC and MBC of each sample. Compounds **2, 4, 6** and ciprofloxacin were used in the time-kill dynamic experiment. The final concentration of DMSO was 1%. A control sample was made using DMSO 1% and the inoculum. At each incubation time point, liquids (50 µL) were removed from the test solution for ten-fold serial dilution. Thereafter, a 100 µL liquid from each dilution was spread on the surface of the MHA plates and incubated at 37 °C for 24h, and the number of CFU/mL was counted. Experiments were carried out in triplicate. Time-kill curves were constructed by plotting the surviving $\log_{10}$ of number of CFU/mL against time (hours).

2.6. Antioxidant assay

2.6.1. DPPH free radical scavenging assay

The free radical scavenging activity of the MeOH extract as well as some of its isolated compounds was performed according to described methods (Djouossi et al., 2015). Briefly, the test samples, prior dissolved in DMSO (SIGMA) beforehand, were mixed with a 20 mg/L 2,2-diphenyl-1-picryl-hydrazyl (DPPH) methanol solution, to give final concentrations of 1, 10, 20, 40, 80, 160, 320, 640 and 1280 µg/mL. After 30 min at room temperature, the absorbance values were measured at 517 nm and converted into percentage of antioxidant activity. L-ascorbic acid was used as a standard control. The percentage of decolouration of DPPH (%) was calculated as follows:

$$\% \text{ decolouration of DPPH} = \frac{(\text{Absorbance of control} - \text{Absorbance of test sample}) \times 100}{\text{Absorbance of control}}$$

The radical scavenging percentages were plotted against the logarithmic values of the concentration of test samples and a linear regression curve was established in order to

calculate the EC_{50} (µg/mL), which is the amount of sample necessary to inhibit by 50% the absorbance of free radical DPPH. All the analyses were carried out in triplicate.

2.6.2. Trolox equivalent antioxidant capacity (TEAC) assay.

The TEAC test was done as previously described (Rice-Evans and Miller, 1994) with slight modifications. In a quartz cuvette, to 950 µL acetate buffer (pH =5.0, 100 mM), the following were added: 20 µL laccase (1 mM stock solution), 20 µL test sample, 10 µL ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)) (74 mM stock solution). The laccase were purified from *Sclerotinia sclerotiorum* according to the protocol described (Mot*et al.*, 2012). The sample concentrations in the assay mixture were 400, 200, 100, 10 µg/mL for the MeOH extract and 20 µg/mL for the isolated compounds. The content of the generated $ABTS^{\bullet+}$ radical was measured at 420 nm after 230 s reaction time and was converted to gallic acid equivalents by the use of a calibration curve (Pearson's correlation coefficient: $r = 0.996$) constructed with 0, 4, 10, 14, 28, 56, 84 µM gallic acid standards rather than Trolox. Experiments were done in triplicate.

2.7. Statistical analysis

Data were analyzed by one-way analysis of variance followed by Waller-Duncan Post Hoc test. The experimental results were expressed as the mean ± Standard Deviation (SD). Differences between groups were considered significant when $p < 0.05$. All analyses were performed using the Statistical Package for Social Sciences (SPSS, version 12.0) software.

3. Results and discussion

3.1. Phytochemical analysis

The structures of the isolated compounds were established using spectroscopic analysis, especially, NMR spectra in conjunction with 2D experiments, COSY, TOCSY, HSQC, HMBC and direct comparison with published information. The nine compounds isolated from the leaves of *M. buchananii* (Fig. 1) were identified as quercetin-3-*O*-*α*-L-arabinopyranoside **1** (Kazuma et al., 2003), epicatechin **2** (Petrus et al., 2012), quercetin **3** (Hossain et al., 2006), kaempferol-3-*O*-*β*-D-rhamnopyranoside **4** (Zhang et al., 2003), quercetin-3-*O*-*α*-L-rhamnopyranoside **5** (Markam et al., 1978), quercetin-3-*O*-*β*-D-glucopyanoside **6** (Xiang et al., 2008), epigallocatechin **7** (Zan et al., 2013), kaempferol **8** (Sarin et Sharma, 2012) and quercetin-3-*O*-[*α*-L-rhamnopyranosyl-(1→6)-*β*-D-glucopyranoside] **9** (Kazuma et al., 2000). These compounds together with the extracts were tested for their antibacterial and antioxidant activities and the results are reported in Table 1 and Fig. 2 - 4.

3.2. Antibacterial activity

The antibacterial activity of the MeOH, *n*-BuOH, EtOAc, and hexane extracts as well as their isolated compounds were examined by microdilution susceptibility assay against six bacterial strains selected on the basis of their relevance as human pathogens. The experiments revealed that the extracts and isolated compounds exhibited variable MICs and significant antimicrobial activity, depending on the microbial strains (Table 1). The MIC values of the extracts ranged from 32 to 512 µg/mL. No activity was noted with hexane extract on *Vibrio cholerae* SG24 (1) and *Vibrio cholerae* NB2 at concentrations up to 512 µg/mL while the most sensitive bacterial strains were found to be *Shigella flexneri* and *Staphylococcus aureus.* EtOAc extract (MIC = 32-256 µg/mL) was the most active extract followed in decreasing order by *n*-BuOH (MIC = 64-256 µg/mL), MeOH (MIC = 128 - 512 µg/mL) and hexane (MIC = 512 - >512 µg/mL) extracts. This observation suggests that the crude methanol extract contains several antibacterial principles with different polarities. Phytochemicals are routinely

368 classified as antimicrobials on the basis of susceptibility tests that produce MIC in the range
369 of 100 to 1000 µg/mL (Simões et al., 2009). Activity is considered to be significant if MIC
370 values are below 100 µg/mL for crude extract and moderate when the MIC values vary from
371 100 to 625 µg/ml (Kuete, 2010). Therefore, the activities recorded with the *n*-BuOH fraction
372 on *Shigella flexneri* and *Staphylococcus aureus* and with EtOAc fraction on *Vibrio cholerae*
373 2, *Shigella flexneri* and *Staphylococcus aureus* can be considered as important.

374 The lowest MIC value of 16 µg/mL was recorded on *Staphylococcus aureus* with
375 compounds **6** and on *Escherichia coli* with compound **8**, whereas the lowest MBC value of 32
376 µg/mL was obtained on *Staphylococcus aureus* with compounds **6** and **2** and on *Shigella*
377 *flexneri* with compound **6**. However, the highest MIC value of 512 µg/mL was recorded on
378 *Vibrio cholerae* SG24 (1) and *Vibrio cholerae* CO6 with MeOH extract, and the highest MBC
379 value of 512 µg/mL was obtained on *Vibrio cholerae* SG24 (1) with the MeOH and EtOAc
380 extracts and on *Vibrio cholerae* CO6 and *Vibrio cholerae* NB2 with the MeOH extract. A
381 lower MBC/MIC (≤4) value signifies that a minimum amount of plant extracts/pure
382 compounds is used to kill the microbial species, whereas, a higher values signifies the use of
383 comparatively more amount of sample for the control of any microorganism (Djouossi et al.,
384 2015).

385 The strains of *V. cholerae* NB2, PC2 (Bag et al., 2008; Thakurta et al., 2007) and
386 *Shigella flexneri* (Acharyya et al., 2015) included in the present study were MDR clinical
387 isolates and these were resistant to commonly used drugs such as ampicillin, streptomycin,
388 tetracycline, nalidixic acid, furazolidone, *co*-trimoxazole, etc. However, these bacterial strains
389 were found to be sensitive to most of the tested samples, suggesting that their administration
390 may represent an alternative treatment against the *V. cholerae*, the causative agent of dreadful
391 disease cholera and *S. flexneri*, the causative agent of shigellosis. Taking into account the
392 medical importance of the tested bacteria, this result can be considered as promising in the

perspective of new antibacterial drugs development. Although flavonoid compounds have been reported to possess antibacterial activity (Garcia et al., 2012; Djouossi et al., 2015), no study has been reported on the activity of these compounds against these types of MDR pathogenic strains.

With regard to the structure-activity relationship analysis, the eight flavonoids showed different degrees of antibacterial activity. Compounds **2** (MIC = 32 to 128 µg/mL) and **6** (MIC = 16 to 64 µg/mL) showed the largest antibacterial activities with the best MIC (16 µg/mL) recorded with compound **6** on *Staphylococcus aureus*. These observations show that the sugar moieties and hydroxyl groups should be responsible for the difference in the observed activity. The mechanism of the active compounds (**1-9**) is still to be studied; nevertheless, their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls. More lipophilic flavonoids may also disrupt microbial membranes (Cowan, 1999). For example, (-)-epigallocatechin gallate inhibit cytoplasmic membrane function, whereas the activity of quercetin has been at least partially attributed to the inhibition of DNA gyrase (Cowan, 1999; Fowler et al., 2011).

3.3. The time-kill kinetic study

The time-kill kinetic study for compounds **2**, **4**, and **6** against *Vibrio cholerae* SG24 (1) (as a function of incubation time) is shown in Fig. 2. It can be noted that significant reduction of the bacterial population is observed with the tested compounds and ciprofloxacin at concentrations corresponding to their MIC, MBC and 2MBC values. At MBC/2MBC values, all the bacterial population was completely killed after 6 h of incubation with ciprofloxacin, compounds **2** and **6** while after 10 h of incubation, there was no observed colony at MIC values with ciprofloxacin and compound **4**.

3.4. Antioxidant activity

Free-radical-scavenging activities of *M. buchananii* extracts and their isolated compounds were assessed by DPPH· and ABTS·+. The results were expressed as gallic acid equivalent antioxidant capacity of tested samples (Fig. 3) and as equivalent concentrations of test samples scavenging 50% of DPPH radical (Fig. 4). Both DPPH· and ABTS·+ measure reductions of radical solutions in the presence of a hydrogen-donating antioxidant. The results of both assays found compounds **3** (EC_{50} = 1.38 µg/mL; TEAC= 89.69 µg/mL), **5** (EC_{50} = 1.56 µg/mL; TEAC= 90.93 µg/mL) and **6** (EC_{50} = 1.42 µg/mL; TEAC= 89.76 µg/mL) to exhibit the most activity and compound **7** (EC_{50} = 107.56 µg/mL; TEAC= 44.98 µg/mL) to exhibit the least activity. The results of the DPPH and ABTS free-radical-scavenging activities are not in the same order for the extracts. This difference in the activity may be due to the presence of potent molecule (s) in some extracts which is more capable of quenching one particular radical than another. The free-radical activity of the extracts can be explained by the presence of phenolic substances. Similarly, previous reports have shown phenolic compounds to contribute significantly to the antioxidant activity of medicinal plants (Lim et al., 2009; Zhao et al., 2010; Djouossi et al., 2015). Phenolic compounds such as flavonoids are known to be potential antioxidant due to their ability to scavenge free radicals and active oxygen species such as singlet oxygen, superoxide anion radical and hydroxyl radicals (Hall and Cupett, 1997; Pietta et al., 1998).

Conclusion

Results obtained from this study may help to exploit the use of the *M. buchananii* leaf extracts and some of their flavonoid contents as pharmacological ingredients for promoting health, especially for cholera/shigellosis and chronic diseases associated with oxidative stress.

Acknowledgements

The authors gratefully acknowledge financial support from the research grant committees of both the University of Dschang and the Cameroonian Ministry of Higher Education.

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588
589 **Glossary**
590

591 *^{13}C-NMR:* thirteen Carbon Nuclear Magnetic Resonance

592 *^{1}H NMR:* Proton Nuclear Magnetic Resonance

593 *2D NMR:* Two-dimension Nuclear Magnetic Resonance

594 *ABTS*: 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)

595 *ATCC:* American Type Culture Collection

596 *CC:* Column Chromatography

597 *COSY*: Correlation Spectroscopy

598 *DMSO:* Dimethylsulfoxide

599 *DPPH:* 1,1-diphenyl-2-picrylhydrazyl radical

600 EC_{50}: Concentration scavenging 50 % DPPH radicals

601 *EtOAc*: Ethyl acetate

602 *HMBC:* Heteronuclear Multiple Bond Connectivities

603 *HR-EI-MS:* High Resolution Electron Impact Mass Spectrometry

604 *HR-TOFESIMS:* High-resolution time of flight electrospray ionization mass spectrometry

605 *HSQC:* The Heteronuclear Single Quantum Coherence

606 *IP:* Institut Pasteur

607 *IR:* Infra-red

608 *MBC:* Minimum bactericidal concentration

609 *MeOH*: Methanol

610 *MHA:* Mueller Hinton agar

611 *MHB:* Mueller Hinton broth

612 *MIC:* Minimum inhibitory concentration

613 *NA:* Nutrient agar

614 *n-BuOH*: *n*-Butanol

615 *NMR:* Nuclear Magnetic Resonance

Rf: Retention factor

ROESY: Rotating-Frame NOE Spectroscopy

SRF/CAM: Section de réserve forestière du Cameroun

TEAC: Trolox equivalent antioxidant capacity

TLC: Thin Layer Chromatography

TMS: Tetramethylsilane

TOCSY: Total Correlation Spectroscopy

UV: Ultra-violet

Figure Legends

Fig. 1. Chemical structures of flavonoids isolated (**1-9**) from the leaves of *Maytenusbuchananii*. **1**: quercetin-3-*O*-*α*-L-arabinopyranoside; **2:** epicatechin; **3:**quercetin; **4:** kaempferol-3-*O*-*β*-D-rhamnopyranoside; **5:** quercetrin-3-*O*-*α*-L-rhamnopyranoside; **6:** quercetin-3-*O*-*β*-D-glucopyanoside; **7:** epigallocatechin; **8:** kaempferol and **9:** Quercetin-3-*O*-[*α*-L-rhamnopyranosyl-(1→6)-*β*-D-glucopyranoside].

Fig. 2. Survival curves for *Vibrio cholerae* SG24 (1) cells exposed to the compounds **2**, **4**, **6**, and ciprofloxacin. Control: MHB medium with DMSO 1% + inoculums.

Fig. 3. Gallic acid equivalent antioxidant capacity (TEAC; µg/mL) of tested samples.

Bars represent the mean ± SD of three independent experiments carried out in triplicate. Letters a-i indicate significant differences between samples according to one way ANOVA and Waller Duncan test; $p<0.05$.

Fig. 4. Equivalent concentrations of test samples scavenging 50% of DPPH radical (EC_{50}).

Bars represent the mean ± SD of three independent experiments carried out in triplicate. Letters a-h indicate significant differences between samples according to one way ANOVA and Waller Duncan test; $p<0.05$.

639 **Table 1**

640 Antibacterial activity (MIC and MBC in µg/mL) of extracts, isolated compounds and
641 reference antibacterial drugs.

Extracts/ Compounds	Inhibition parameters	*Vibrio cholerae* SG24 (1)	*Vibrio cholerae* CO6	*Vibrio cholerae* NB2	*Vibrio cholerae* PC2	*Shigella flexneri* SDINT	*Staphylococcus aureus ATCC 25923*
MeOH extract	MIC	512	512	256	256	128	128
	MBC	>512	512	512	512	256	128
	MBC/MIC	/	1	2	2	2	1
n-BuOH extract	MIC	256	128	128	128	64	64
	MBC	256	256	256	256	64	64
	MBC/MIC	1	2	2	2	1	1
EtOAc extract	MIC	128	256	128	64	64	32
	MBC	128	512	128	64	64	64
	MBC/MIC	1	2	1	1	1	2
Hexane extract	MIC	>512	512	>512	512	512	512
	MBC	/	>512	/	>512	>512	>512
	MBC/MIC	/	/	/	/	/	/
1	MIC	256	128	256	256	128	64
	MBC	256	256	>256	256	128	64
	MBC/MIC	1	2	/	1	1	1
2	MIC	32	64	64	128	64	32
	MBC	64	64	128	256	128	32
	MBC/MIC	2	1	2	2	2	1
3	MIC	128	64	128	128	64	64
	MBC	256	128	128	128	128	64
	MBC/MIC	2	2	1	1	2	1
4	MIC	128	128	64	128	64	64
	MBC	256	128	128	256	64	128
	MBC/MIC	2	1	2	2	1	2
5	MIC	128	128	64	128	128	64
	MBC	256	256	128	128	256	128
	MBC/MIC	2	2	2	1	2	2
6	MIC	64	64	64	64	32	16
	MBC	128	128	128	64	32	32
	MBC/MIC	2	2	2	1	1	2
7	MIC	256	128	128	128	64	64
	MBC	>256	128	>256	256	64	64
	MBC/MIC	/	1	/	2	1	1

8	MIC	128	256	256	128	128	64
	MBC	256	256	>256	>256	256	128
	MBC/MIC	2	1	/	/	2	2
9	MIC	32	32	64	64	64	32
	MBC	64	32	64	128	64	32
	MBC/MIC	2	1	1	2	1	1
Ampicillin	MIC	16	16	>512	>512	>512	8
	MBC	16	16	>512	>512	>512	8
	MBC/MIC	1	1	/	/	/	1
Ciprofloxacin	MIC	8	8	16	16	16	2
	MBC	8	8	16	16	16	2
	MBC/MIC	1	1	1	1	1	1

642 /: not determined; MIC: Minimum Inhibitory Concentration; MBC Minimum Bactericidal Concentration.
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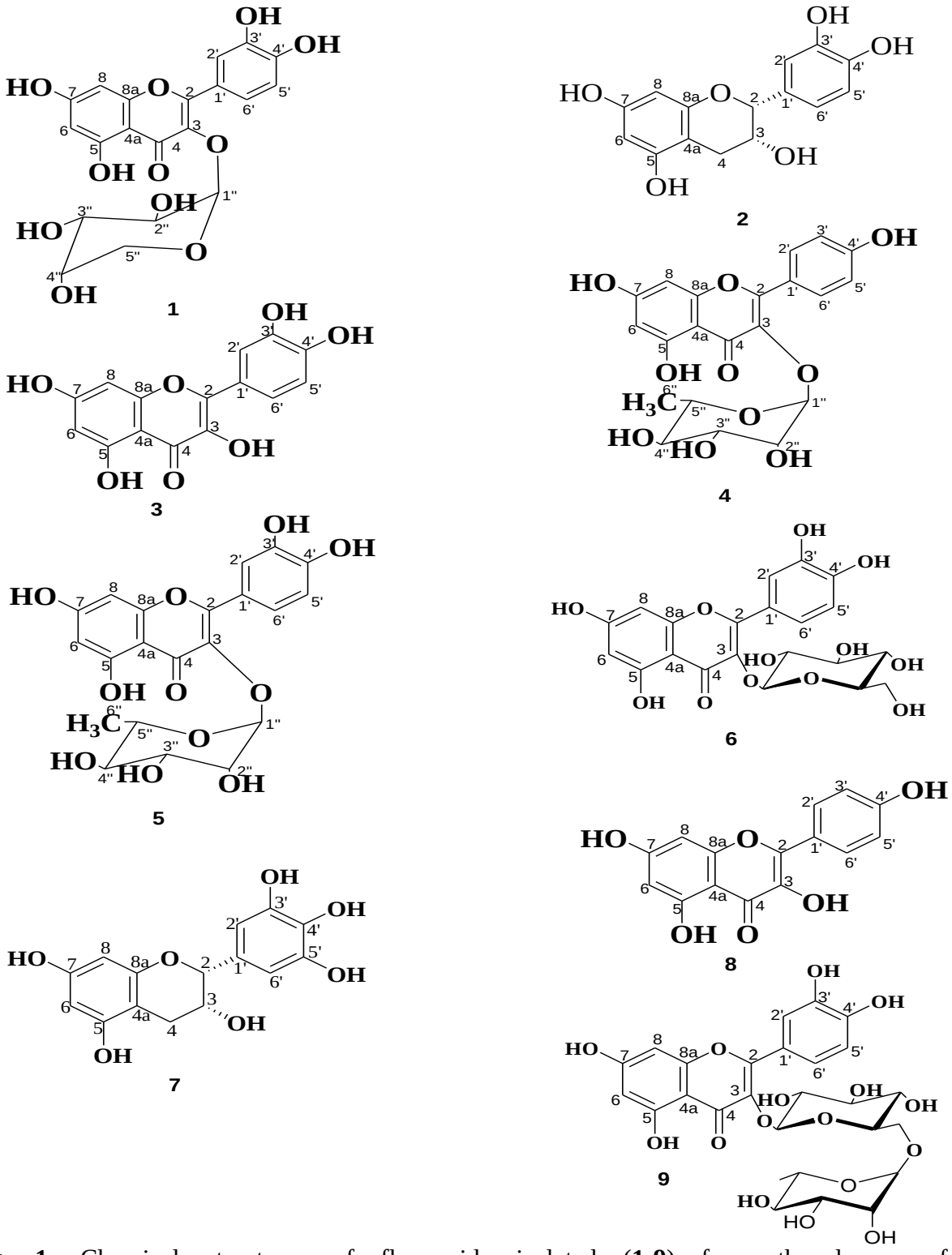

Fig. 1. Chemical structures of flavonoids isolated (**1-9**) from the leaves of *Maytenusbuchananii*. **1**: quercetin-3-*O*-*α*-L-arabinopyranoside; **2:** epicatechin; **3:**quercetin; **4:** kaempferol-3-*O*-*β*-D-rhamnopyranoside; **5:** quercetrin-3-*O*-*α*-L-rhamnopyranoside; **6:**

651 quercetin-3-*O*-*β*-D-glucopyanoside; **7:** epigallocatechin; **8:** kaempferol and **9:** Quercetin-3-*O*-

652 [*α*-L-rhamnopyranosyl-(1→6)-*β*-D-glucopyranoside].

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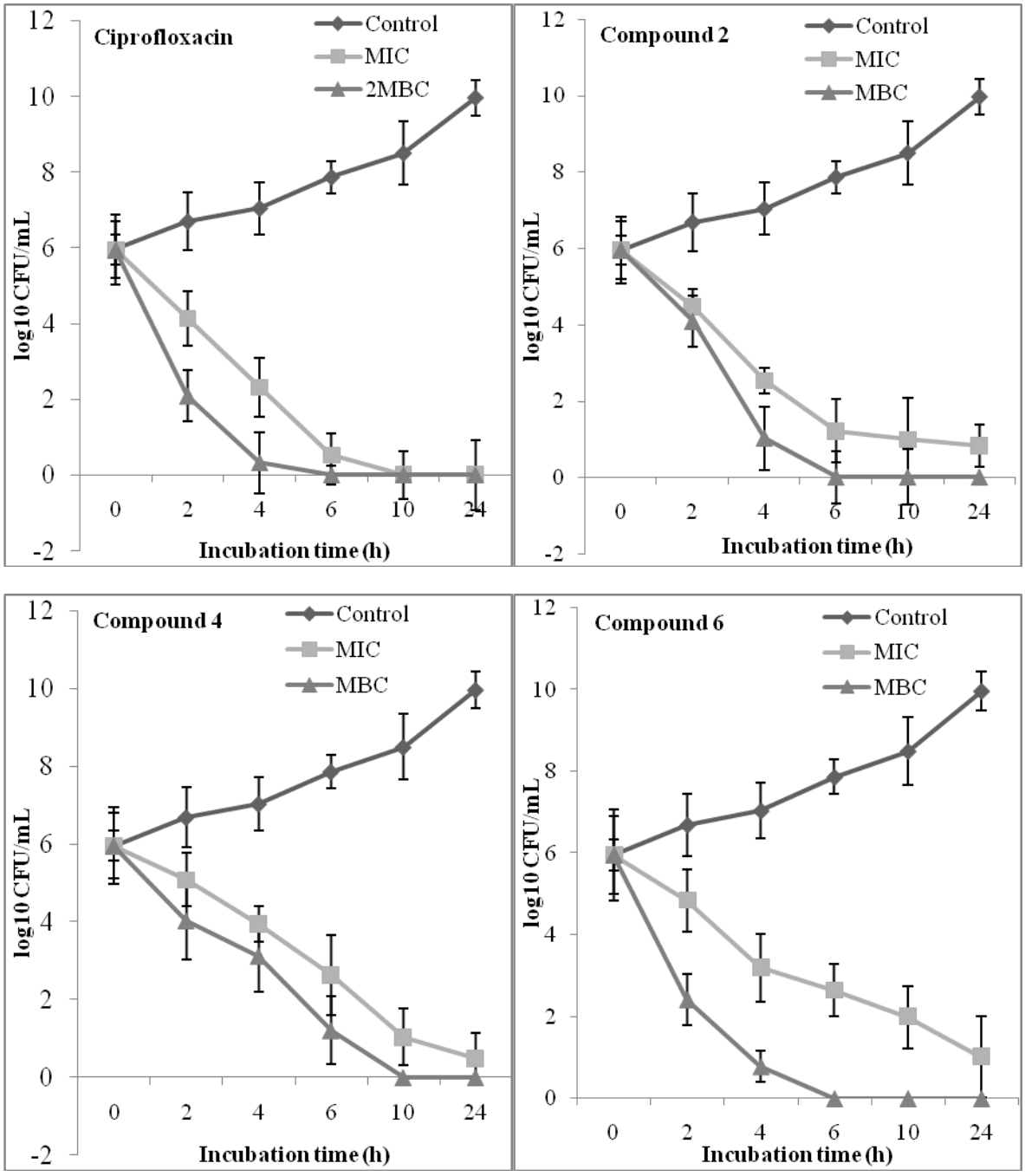

Fig. 2. Survival curves for *Vibrio cholerae* SG24 (1) cells exposed to the compounds **2**, **4**, **6**, and ciprofloxacin. Control: MHB medium with DMSO 1% + inoculums.

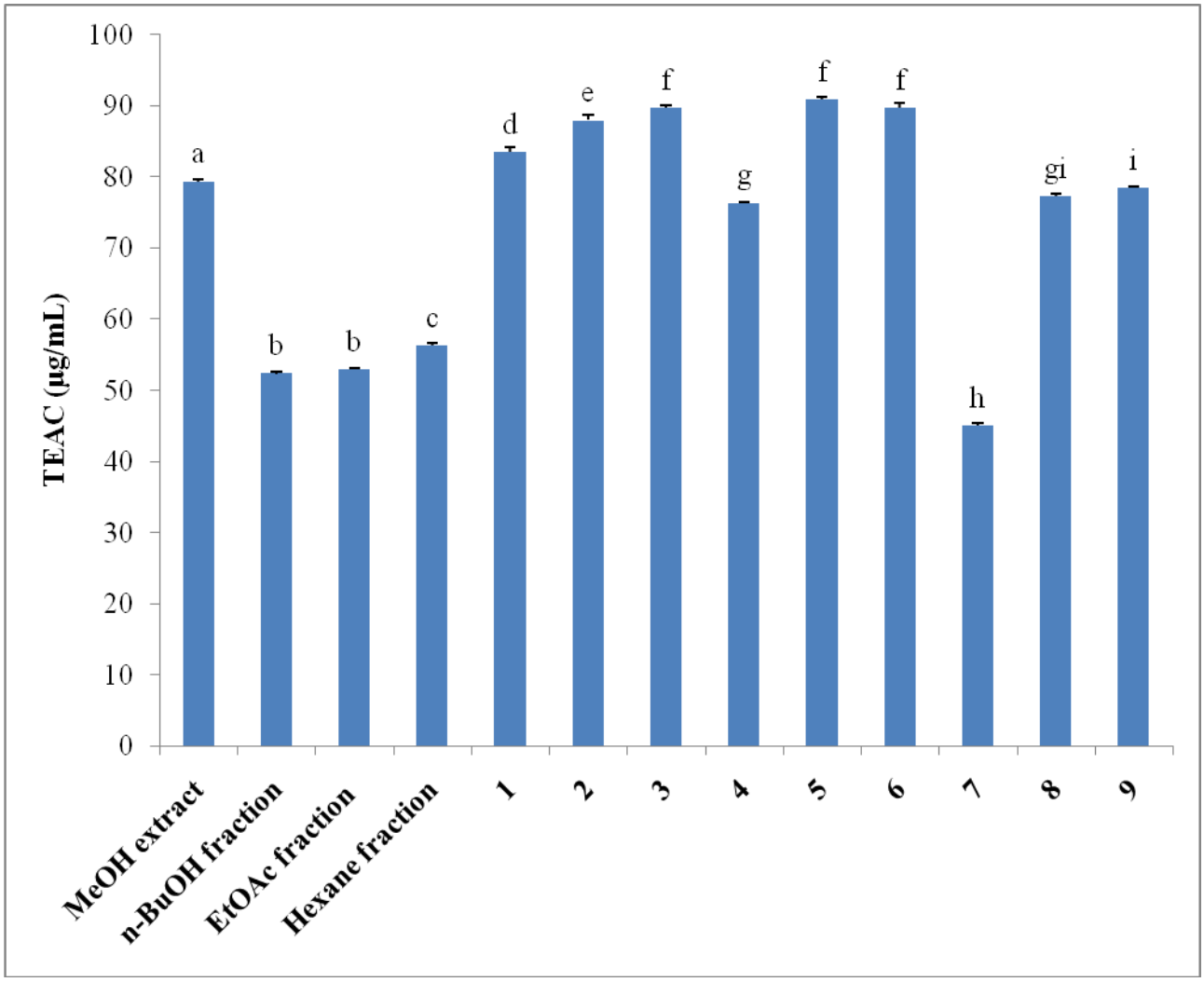

Fig. 3. Gallic acid equivalent antioxidant capacity (TEAC; µg/mL) of tested samples. Bars represent the mean ± SD of three independent experiments carried out in triplicate. Letters a-i indicate significant differences between samples according to one way ANOVA and Waller Duncan test; $p<0.05$.

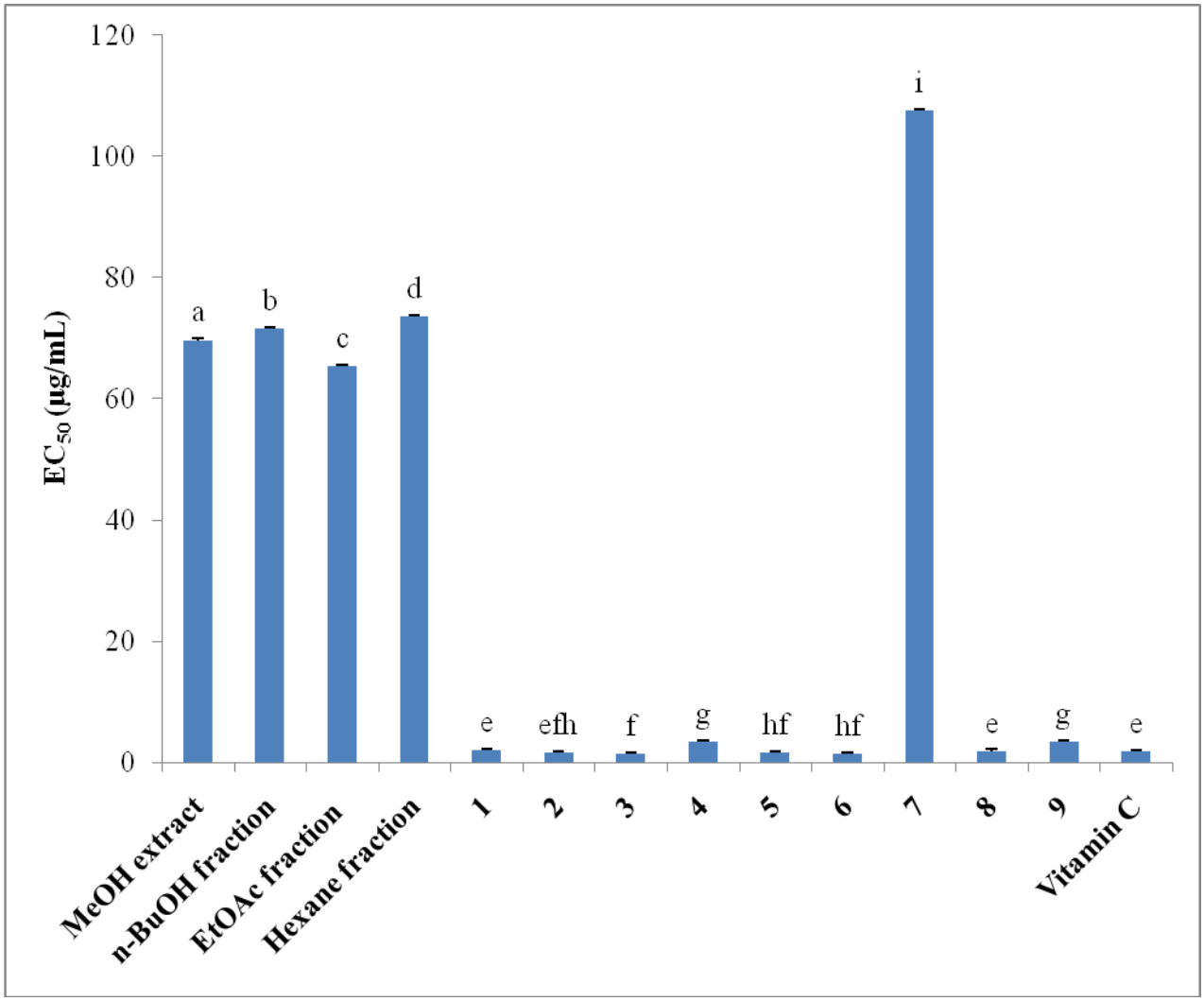

Fig. 4. Equivalent concentrations of test samples scavenging 50% of DPPH radical (EC_{50}). Bars represent the mean ± SD of three independent experiments carried out in triplicate. Letters a-h indicate significant differences between samples according to one way ANOVA and Waller Duncan test; $p<0.05$.