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Flavonoids from *Maytenus buchananii* as potential cholera chemotherapeutic agents

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32   **ABSTRACT**

33   *Maytenus buchananii* is worldwide distributed small evergreen tree traditionally used

34   as anti-tumor, anti-ulcer, anti-inflammatory, and as antimicrobial. The column

35   chromatography of the MeOH extract of *M. buchananii* leaves followed by purification of

36   different fractions led to the isolation of nine known flavonoids. Their structures were

37   elucidated on the basis of spectra analysis, and by comparison with those from the literature.

38   The antibacterial activity was assessed by performing minimum inhibitory concentration

39   (MIC), minimum bactericidal concentration (MBC) and the time-kill kinetic study while the

40   antioxidant activity was determined by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) and trolox

41   equivalent antioxidant capacity (TEAC) assays. All of the extracts/compounds showed

42   antibacterial activity against the strains of Gram-positive bacteria, *Staphylococcus aureus*, and

43   Gram-negative multi-drug resistance bacteria, *Vibrio cholerae* (causative agent of cholera)

44   and *Shigella flexneri* (causative agent of shigellosis). Compounds **2** (MIC = 32 to 128 μg/mL)

45   and **6** (MIC = 16 to 64 μg/mL) showed the largest antibacterial activities with the best MIC

46   (16 μg/mL) recorded with compound **6** on *Staphylococcus aureus*. The results of both DPPH

47 and TEAC assays found compounds **3** (EC_{50} = 1.38 µg/mL; TEAC= 89.69 µg/mL), **5** (EC_{50} =

48 1.56 µg/mL; TEAC= 90.93 µg/mL) and **6** (EC_{50} = 1.42 µg/mL; TEAC= 89.76 µg/mL) to

49 exhibit the most antioxidant activity. Results obtained from this study may help to exploit the

50 use of the *M. buchananii* leaf extracts and some of their flavonoid compounds as

51 pharmacological ingredients for promoting health, especially for cholera/shigellosis and

52 chronic diseases associated with oxidative stress.

53

54 *Keywords*: *Maytenus buchananii*; Celastraceae; leaf extract; phytochemical analysis;

55 flavonoids; antioxidant; antimicrobial; time-kill study.

56

57 **1. Introduction**

58   Acute watery diarrhea accounts for 80% of the cases (death account for 50%) in the

59 developing world (Tullock and Richards, 1993). Among the diarrheal diseases, cholera is a

60 serious epidemic disease caused by the gram-negative bacterium *Vibrio cholerae* (Nair et al.,

61 1994). *Vibrio cholerae*, serotypes O1 and O139 has ability to produce an enterotoxin, cholera

62 toxin that is a major determinant of virulence for cholera. Among the other virulence factors,

63 ElTor hemolysin produced by *Vibrio cholerae* is also reportedly a potent toxin with both

64 enterotoxic and cytotoxic activities (Ichinose et al., 1987; Ramamurthy et al., 1993). Some

65 drugs such as racecadotril and loperamide used to treat the secretary diarrhea, have side

66 effects such as bronchopasm, vomiting and fever, and loperamide should not be administrated

67 to children below 6 years of age, patients with constipation, and intestinal obstruction (Roge

68 et al., 1993; Salazar et al., 2000). Emergence of multiply drug-resistant *Vibrio cholerae* is a

69 serious clinical problem in the treatment and containment of the disease, as reflected by the

70 increase in the fatality rate from 1% to 5.3% after the emergence of drug-resistant strains in

71 Guinea-Bissau during the 1996–1997 epidemic of cholera (Dalsgaard et al., 1999). The

72 increasing incidence of drug-resistant pathogens has drawn the attention of the pharmaceutical

73 and scientific communities towards studies on the potential antimicrobial activity of plant-

74 derived products, an untapped source of antimicrobial chemotypes, which are used in

75 traditional medicine in different countries. *Maytenus buchananii* (Loes.) R. Wilczek

76 belonging to the Celastraceae family is wordwide distributed small evergreen tree 2-12 m

77 high, with paired spinesup to 2.8 mm long. It has been reported that plants of this genus are

78 widely used in folk medicine as anti-tumor, anti-asthmatic, anti-ulcer, treatment of stomach

79 problems, anti-inflammatory, analgesic and as antimicrobial (Ghazanfar, 1994; Muhammad et

80 al., 2000; Orabi et al., 2001; Al Haidari et al., 2002). The biological activities associated to

81 *Maytenus* species have been assigned to different classes of secondary metabolites such as

82 triterpenes (Sannomiya et al., 1998; El Tahir et al., 2001; Lindsey et al., 2006; Martucciello et

83 al., 2010), phenolics glucosides (Da Silva et al., 2008), alkaloids (Corsino et al., 1998a; Orabi

84 et al., 2001) and flavonoids (Vilegas et al., 1999; Lindsey et al., 2006; Dias et al., 2007).

85 Flavonoids, previously called bioflavonoids and included in aromatic compounds, are

86 important constituents of plants. The basic structural feature of flavonoid compounds is the 2-

87 phenylbenzopyrane or flavane nucleus, consisting of two benzene rings linked through a

88 heterocyclic pyrane ring. In total, there are 14 classes of flavonoids, differentiated on the basis

89 of the chemical nature and position of substituents on the different rings. For centuries,

90 preparations containing these compounds as the principal physiologically active constituents

91 have been used to treat human diseases such as infections associated with bacteria and those

92 related to oxidative stress. Many investigations revealed that flavonoids content contribute to

93 the antimicrobial (Araujo et al., 2011; Garcia et al., 2012; Djouossi et al., 2015) and

94 antioxidant (Pietta et al., 1998; Djouossi et al., 2015) activities of plants. In the course of our

95 continuing search for secondary metabolites of biological importance from Cameroonian

96   medicinal plants, we evaluate the antibacterial and antioxidant activities of extracts and

97   flavonoids from the leaves of *M. buchananii*.

98

99   **2. Materials and methods**

100   2.1. General

101   Optical rotations were measured on a Perkin-Elmer 341 polarimeter. ^{1}H and ^{13}C-NMR

102   spectra were recorded on a Bruker Avance III 600 spectrometer equipped with a cryoplatform

103   (^{1}H at 600 MHz and ^{13}C at 150 MHz). 2D-NMR experiments were performed using standard

104   Bruker microprograms (Xwin-NMR version 2.1 software). Chemical shifts (δ) are reported in

105   parts per million (ppm) with the solvent signals as reference relative to TMS (δ=0) as internal

106   standard, while the coupling constants (*J* values) are given in Hertz (Hz). The IR spectra were

107   recorded with a Shimadzu FT-IR-8400S spectrophotometer. UV spectra were determined as

108   methanol solution with a Cary 50 UV/VIS Spectrophotometer. HR-TOFESIMS experiments

109   were performed using a Micromass Q-TOF micro instrument (Manchester, UK) with an

110   electrospray source. The samples were introduced by direct infusion in a solution of MeOH at

111   a rate of 5 µL/min. Column chromatography (CC) was performed on silica gel 60 (70-230

112   mesh, Merck) and gel permeation on Sephadex LH-20 while TLC was carried out on silica

113   gel GF$_{254}$ pre-coated plates with detection accomplished by spraying with 50% H_2SO_4

114   followed by heating at 100 °C, or by visualizing with an UV lamp at 254 and 365 nm.

115

116   *2.2. Plant material*

117   The leaves of *Maytenus buchananii* was collected at Dschang, Menoua Division, West

118   Region of Cameroon, in March 2012. Authentication was done by Victor Nana, a botanist of

119   the Cameroon National Herbarium, Yaoundé, where a voucher specimen (N^o

120   12659/SFR/CAM) is deposited.

121

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

146   fractions (75 mL each) were collected, yielding six sub-fractions. Purification of sub-fractions

147   [14-24] on silica gel with hexane-EtOAc (2:3) gave compound **7** (12 mg), while sub-fractions

148   [41-50] and [106-121] were passed through Sephadex LH-20 column chromatography using

149   MeOH and purified on silica gel column chromatography with hexane-EtOAc (3:7) and (1:9)

150   respectively to afford compounds **5** (14 mg) and **6** (24 mg) respectively.

151   50 g of the gum were repeatedly subjected to Sephadex LH-20 column chromatography

152   using MeOH to separate compounds (Extract) from the trail (Rest). This extract (35 g) was

153   purified by column chromatography over silica gel with EtOAc. Fractions of 100 mL were

154   collected and combined on the basis of their TLC profiles to give six fractions noted FG1 to

155   FG6. Fraction FG1 was purified on silica gel column chromatography eluted with hexane-

156   EtOAc (4:1) and fractions (25 mL each) were collected to give four sub-fractions. Purification

157   of sub-fraction [5] gave compound **8** (17 mg). Fraction F3G was purified over silica gel

158   column chromatography eluted with the mixture EtOAc-MeOH-H_2O (95-3-2) to give

159   compound **9** (20 mg).

160

161   *2.4. Structural identification of the isolated compounds*

162   Samples for NMR experiments were dissolved in CD_3OD on a BRUKER Avance DRX

163   600 Spectrometer (600MHz for ^{1}H and 150MHz for ^{13}C). Column chromatography was

164   performed on silica gel 60 (70-230 mesh, Merck) and sephadex LH-20. Fractions were

165   monitored by TLC using Merck pre-coated silica gel sheets (60 F_{254}), and spots were

166   visualized under UV light (254 and 365 nm) and by spraying with 50% H_2SO_4 and heating at

167   100 °C. 1D and 2D-NMR experiments (COSY, TOCSY, ROESY, HSQC-*Jmod*, and HMBC)

168   were performed using standard Bruker pulse programs (XW in NMR version 2.1).

169   Quercetin-3-*O*-α-L-arabinopyranoside **1**: yellow powder. UV: 250, 279, 260, 375 nm. IR

170   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$_3$OD, 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$_3$OD, 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$_3$OD, 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$_3$OD, 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$_3$OD, 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$_3$OD, 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$_3$OD, 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$_3$OD, 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$_3$OD, 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$_3$OD, 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$_3$OD, 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

220 (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-

221 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

223 (broad), 1620, 1520, 1470, 1380, 1280, 1240. ^{13}C-NMR (CD$_3$OD, 150 MHz) δ: 78.5 (C-2),

224 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-

225 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-

226 NMR (CD$_3$OD, 600 MHz), δ: 4.77 (1H, m, H-2), 4.19 (1H, m, H-3), 2.75 (1H, dd, J = 2.9 and

227 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

228 (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,

230 1658, 1592, 1455, 1350, 1208, 1094. ^{13}C-NMR (CD$_3$OD, 150 MHz) δ: 158.3 (C-2), 137.2 (C-

231 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),

232 123.8 (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

233 (CD$_3$OD, 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,

234 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.

236 UV: 254, 268, 310, 355 nm. IR ν_{max} (KBr, cm^{-1}): 3288, 2987, 1598, 1504, 1460, 1348, 1209,

237 1092. ^{13}C-NMR (CD$_3$OD, 150 MHz) δ:157.5 (C-2), 134.4 (C-3), 178.0 (C-4), 161.6 (C-5),

238 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'),

239 144.4 (C-3'), 148.6 (C-4'), 114.7 (C-5'), 121.6 (C-6') for aglycone; 104.6 (C-1"), 71.7 (C-2"),

240 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-

241 2'''), 70.9 (C-3'''), 72.5 (C-4'''), 68.3 (C-5'''), 16.6 (C-6''') for rhamnosyl sugar. ^{1}H-NMR

242 (CD$_3$OD, 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,

243 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')

244 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

245 (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

246 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,

247 H-4'''), 3.55 (1H, m, H-5'''), 0.95 (3H, m, H-6''') for rhamnosyl sugar.

248

249 *2.5. Antimicrobial assay*

250 *2.5.1. Microorganisms*

251   A total of six bacterial strains were tested for their susceptibility to compounds and

252 these strains were taken from our laboratory collection (kindly provided by Dr. T.

253 Ramamurthy, NICED, Kolkata). Among the clinical strains of *Vibrio cholerae* used in this

254 study, strains NB2 and SG24(1) belonged to O1 and O139 serotypes, respectively. These

255 strains were able to produce cholera toxin and hemolysin (Bag et al., 2008; Thakurta et al.,

256 2007). The other strains used in this study were *V. cholerae* non-O1, non-O139 (strains CO6

257 and PC2) (Bag et al., 2008); and *Shigella flexneri* (Acharyya et al., 2015). The *V. cholerae*

258 non-O1 and non-O139 strains, were positive for hemolysin production but negative for

259 cholera toxin production (Bag et al., 2008). The American Type Culture Collection (ATCC)

260 strain, *Staphylococcus aureus* ATCC 25923, was used for quality control. The bacterial

261 strains were maintained on agar slant at 4 °C and subcultured on a fresh appropriate agar

262 plates 24 h prior to any antibacterial test. The Mueller Hinton Agar (MHA) was used for the

263 activation of bacteria. The Mueller Hinton Broth (MHB) and nutrient agar (Hi-Media) were

264 used for the MIC and MBC determinations respectively.

265

266 *2.5.2. Determination of minimum inhibitory concentration (MIC) and minimum bactericidal*

267 *concentration (MBC)*

268   MIC values were determined by a broth micro-dilution method as described earlier

269 (Nyaa et al., 2009) with slight modifications. Each test sample was dissolved in

270 dimethylsulfoxide (DMSO) and the solution was then added to Mueller Hinton Broth (MHB)

271 for bacteria to give a final concentration of 1024 μg/mL. This was serially diluted twofold to

272 obtain a concentration range of 0.50–1024 μg/mL. Then, 100 μL of each concentration was

273 added in each well (96-well microplate) containing 95 μL of MHB and 5 μL of inoculum for

274 final concentrations varying from 0.25–512 μg/mL. The inoculum was standardized at 1.5

275 $\times 10^6$ CFU/mL by adjusting the optical density to 0.1 at 600 nm using a JENWAY 6105

276 UV/Vis spectrophotometer. The final concentration of DMSO in each well was < 1%

277 [preliminary analyses with 1% (v/v) DMSO did not inhibit the growth of the test organisms].

278 The negative control well consisted of 195 μL of MHB and 5 μL of the standard inoculum.

279 The plates were covered with sterile lids, then agitated to mix the contents of the wells using a

280 plate shaker and incubated at 35 °C for 24 h. The assay was repeated three times. The MIC

281 values of samples were determined by adding 50 μL of a 0.2 mg/mL *p*-iodonitrotetrazolium

282 violet solution followed by incubation at 35 °C for 30 min. Viable microorganisms reduced

283 the yellow dye to a pink color. MIC values were defined as the lowest sample concentrations

284 that prevented this change in color indicating a complete inhibition of microbial growth. For

285 the determination of MBC values, a portion of liquid (5 μL) from each well that showed no

286 growth of microorganism was plated on Mueller Hinton Agar and incubated at 35 °C for 24

287 h. The lowest concentrations that yielded no growth after this subculturing were taken as the

288 MBC values (Tamokou et al., 2011). Ciprofloxacin and ampicillin (Sigma-Aldrich,

289 Steinheim, Germany) were used as positive controls.

290

291 *2.5.3. The time-kill kinetic study (for antimicrobial drugs) against Vibrio cholerae SG24 (1)*

292 Time-kill dynamic assay was performed using broth microdilution method as

293 previously described (Avila et al., 1999) with minor modifications. Cultures of bacteria in

294 MHB (1 × 10^6 CFU/mL) were incubated separately at 37 °C for 0, 2, 4, 6, 10, and 24 hours in

295   the absence (control) and in the presence of the drug/extract at MIC and MBC of each sample.

296   Compounds **2, 4, 6** and ciprofloxacin were used in the time-kill dynamic experiment. The

297   final concentration of DMSO was 1%. A control sample was made using DMSO 1% and the

298   inoculum. At each incubation time point, liquids (50 µL) were removed from the test solution

299   for ten-fold serial dilution. Thereafter, a 100 µL liquid from each dilution was spread on the

300   surface of the MHA plates and incubated at 37 °C for 24h, and the number of CFU/mL was

301   counted. Experiments were carried out in triplicate. Time-kill curves were constructed by

302   plotting the surviving $\log_{10}$ of number of CFU/mL against time (hours).

303

304   *2.6. Antioxidant assay*

305   *2.6.1. DPPH free radical scavenging assay*

306   The free radical scavenging activity of the MeOH extract as well as some of its

307   isolated compounds was performed according to described methods (Djouossi et al., 2015).

308   Briefly, the test samples, prior dissolved in DMSO (SIGMA) beforehand, were mixed with a

309   20 mg/L 2,2-diphenyl-1-picryl-hydrazyl (DPPH) methanol solution, to give final

310   concentrations of 1, 10, 20, 40, 80, 160, 320, 640 and 1280 µg/mL. After 30 min at room

311   temperature, the absorbance values were measured at 517 nm and converted into percentage

312   of antioxidant activity. L-ascorbic acid was used as a standard control. The percentage of

313   decolouration of DPPH (%) was calculated as follows:

314

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

315   % decolouration of DPPH =

316

317   The radical scavenging percentages were plotted against the logarithmic values of the

318   concentration of test samples and a linear regression curve was established in order to

319   calculate the EC_{50} (μg/mL), which is the amount of sample necessary to inhibit by 50% the

320   absorbance of free radical DPPH. All the analyses were carried out in triplicate.

321

322   *2.6.2. Trolox equivalent antioxidant capacity (TEAC) assay.*

323   The TEAC test was done as previously described (Rice-Evans and Miller, 1994) with

324   slight modifications. In a quartz cuvette, to 950 μL acetate buffer (pH =5.0, 100 mM), the

325   following were added: 20 μL laccase (1 mM stock solution), 20 μL test sample, 10 μL ABTS

326   (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)) (74 mM stock solution). The laccase

327   were purified from *Sclerotinia sclerotiorum* according to the protocol described (Mot*et al.*,

328   2012). The sample concentrations in the assay mixture were 400, 200, 100, 10 μg/mL for the

329   MeOH extract and 20 μg/mL for the isolated compounds. The content of the generated

330    $ABTS^{\bullet+}$ radical was measured at 420 nm after 230 s reaction time and was converted to gallic

331   acid equivalents by the use of a calibration curve (Pearson's correlation coefficient: $r = 0.996$)

332   constructed with 0, 4, 10, 14, 28, 56, 84 μM gallic acid standards rather than Trolox.

333   Experiments were done in triplicate.

334

335   *2.7. Statistical analysis*

336   Data were analyzed by one-way analysis of variance followed by Waller-Duncan Post

337   Hoc test. The experimental results were expressed as the mean ± Standard Deviation (SD).

338   Differences between groups were considered significant when $p < 0.05$. All analyses were

339   performed using the Statistical Package for Social Sciences (SPSS, version 12.0) software.

340

341   **3. Results and discussion**

342   *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

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

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

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

393 perspective of new antibacterial drugs development. Although flavonoid compounds have

394 been reported to possess antibacterial activity (Garcia et al., 2012; Djouossi et al., 2015), no

395 study has been reported on the activity of these compounds against these types of MDR

396 pathogenic strains.

397   With regard to the structure-activity relationship analysis, the eight flavonoids showed

398 different degrees of antibacterial activity. Compounds **2** (MIC = 32 to 128 µg/mL) and **6**

399 (MIC = 16 to 64 µg/mL) showed the largest antibacterial activities with the best MIC (16

400 µg/mL) recorded with compound **6** on *Staphylococcus aureus*. These observations show that

401 the sugar moieties and hydroxyl groups should be responsible for the difference in the

402 observed activity. The mechanism of the active compounds (**1-9**) is still to be studied;

403 nevertheless, their activity is probably due to their ability to complex with extracellular and

404 soluble proteins and to complex with bacterial cell walls. More lipophilic flavonoids may also

405 disrupt microbial membranes (Cowan, 1999). For example, (-)-epigallocatechin gallate inhibit

406 cytoplasmic membrane function, whereas the activity of quercetin has been at least partially

407 attributed to the inhibition of DNA gyrase (Cowan, 1999; Fowler et al., 2011).

408

409 *3.3. The time-kill kinetic study*

410   The time-kill kinetic study for compounds **2**, **4**, and **6** against *Vibrio cholerae* SG24

411 (1) (as a function of incubation time) is shown in Fig. 2. It can be noted that significant

412 reduction of the bacterial population is observed with the tested compounds and ciprofloxacin

413 at concentrations corresponding to their MIC, MBC and 2MBC values. At MBC/2MBC

414 values, all the bacterial population was completely killed after 6 h of incubation with

415 ciprofloxacin, compounds **2** and **6** while after 10 h of incubation, there was no observed

416 colony at MIC values with ciprofloxacin and compound **4**.

417

3.4. Antioxidant activity

419   Free-radical-scavenging activities of *M. buchananii* extracts and their isolated compounds

420 were assessed by DPPH· and ABTS·+. The results were expressed as gallic acid equivalent

421 antioxidant capacity of tested samples (Fig. 3) and as equivalent concentrations of test

422 samples scavenging 50% of DPPH radical (Fig. 4). Both DPPH· and ABTS·+ measure

423 reductions of radical solutions in the presence of a hydrogen-donating antioxidant. The results

424 of both assays found compounds **3** (EC_{50} = 1.38 µg/mL; TEAC= 89.69 µg/mL), **5** (EC_{50} =

425 1.56 µg/mL; TEAC= 90.93 µg/mL) and **6** (EC_{50} = 1.42 µg/mL; TEAC= 89.76 µg/mL) to

426 exhibit the most activity and compound **7** (EC_{50} = 107.56 µg/mL; TEAC= 44.98 µg/mL) to

427 exhibit the least activity. The results of the DPPH and ABTS free-radical-scavenging

428 activities are not in the same order for the extracts. This difference in the activity may be due

429 to the presence of potent molecule (s) in some extracts which is more capable of quenching

430 one particular radical than another. The free-radical activity of the extracts can be explained

431 by the presence of phenolic substances. Similarly, previous reports have shown phenolic

432 compounds to contribute significantly to the antioxidant activity of medicinal plants (Lim et

433 al., 2009; Zhao et al., 2010; Djouossi et al., 2015). Phenolic compounds such as flavonoids

434 are known to be potential antioxidant due to their ability to scavenge free radicals and active

435 oxygen species such as singlet oxygen, superoxide anion radical and hydroxyl radicals (Hall

436 and Cupett, 1997; Pietta et al., 1998).

437

Conclusion

439   Results obtained from this study may help to exploit the use of the *M. buchananii* leaf

440 extracts and some of their flavonoid contents as pharmacological ingredients for promoting

441 health, especially for cholera/shigellosis and chronic diseases associated with oxidative stress.

442

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588

589   **Glossary**

590

591   ^{13}*C-NMR:* thirtheen 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

616 *Rf:* Retention factor

617 *ROESY*: Rotating-Frame NOE Spectroscopy

618 SRF/CAM: Section de réserve forestière du Cameroun

619 *TEAC*: Trolox equivalent antioxidant capacity

620 *TLC:* Thin Layer Chromatography

621 *TMS:* Tetramethylsilane

622 *TOCSY*: Total Correlation Spectroscopy

623 *UV:* Ultra-violet

624

625 **Figure Legends**

626 **Fig. 1.** Chemical structures of flavonoids isolated (**1-9**) from the leaves of

627 *Maytenusbuchananii*. **1**: quercetin-3-*O*-*α*-L-arabinopyranoside; **2**: epicatechin; **3:**quercetin; **4:** kaempferol-

628 3-*O*-*β*-D-rhamnopyranoside; **5:** quercetrin-3-*O*-*α*-L-rhamnopyranoside; **6:** quercetin-3-*O*-*β*-D-glucopyanoside;

629 **7:** epigallocatechin; **8:** kaempferol and **9:** Quercetin-3-*O*-[*α*-L-rhamnopyranosyl-(1→6)-*β*-D-glucopyranoside].

630 **Fig. 2.** Survival curves for *Vibrio cholerae* SG24 (1) cells exposed to the compounds **2**, **4**, **6**,

631 and ciprofloxacin. Control: MHB medium with DMSO 1% + inoculums.

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

633 Bars represent the mean ± SD of three independent experiments carried out in triplicate. Letters a-i indicate

634 significant differences between samples according to one way ANOVA and Waller Duncan test; $p < 0.05$.

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

636 Bars represent the mean ± SD of three independent experiments carried out in triplicate. Letters a-h indicate

637 significant differences between samples according to one way ANOVA and Waller Duncan test; $p < 0.05$.

638

Table 1

Antibacterial activity (MIC and MBC in μg/mL) of extracts, isolated compounds and
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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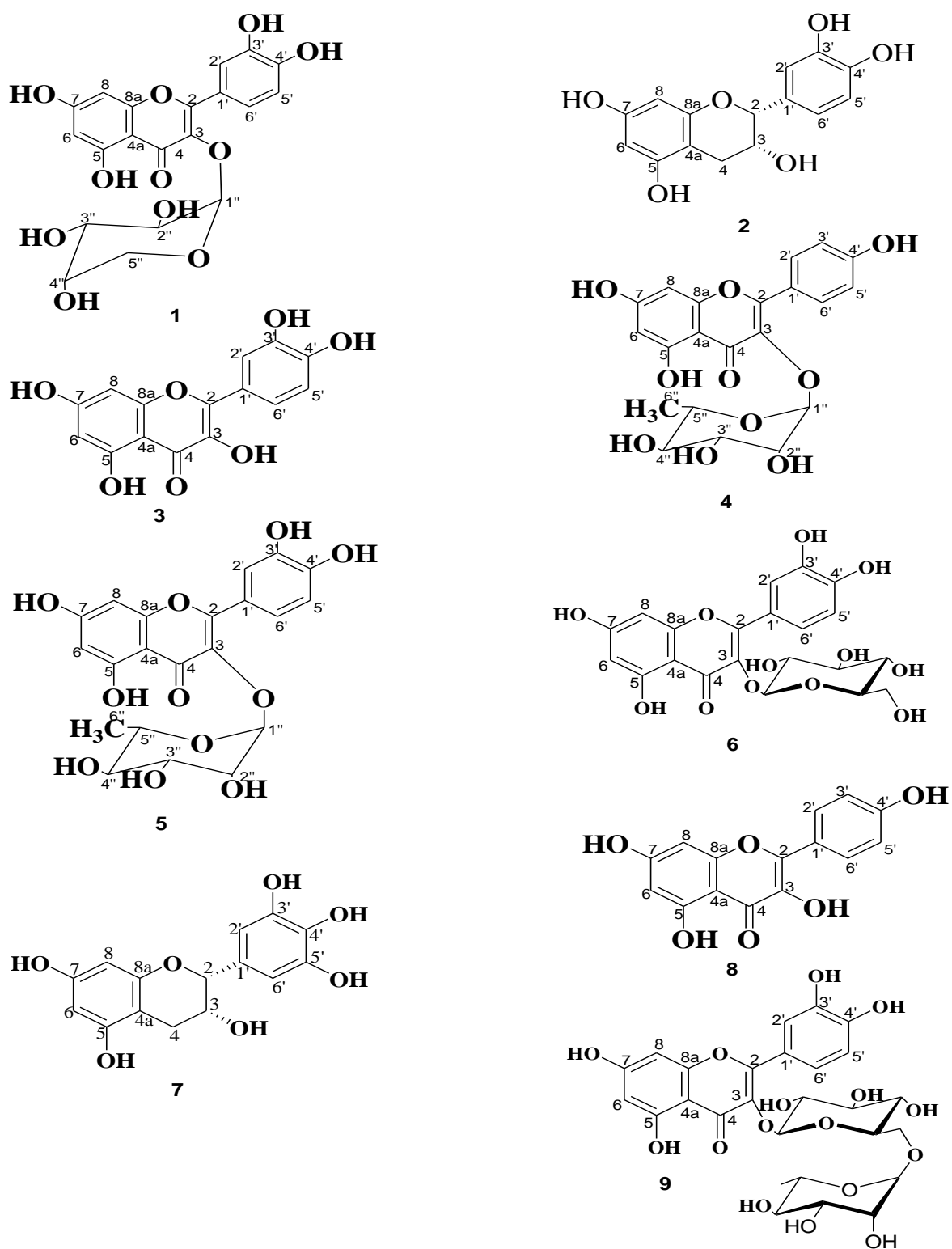
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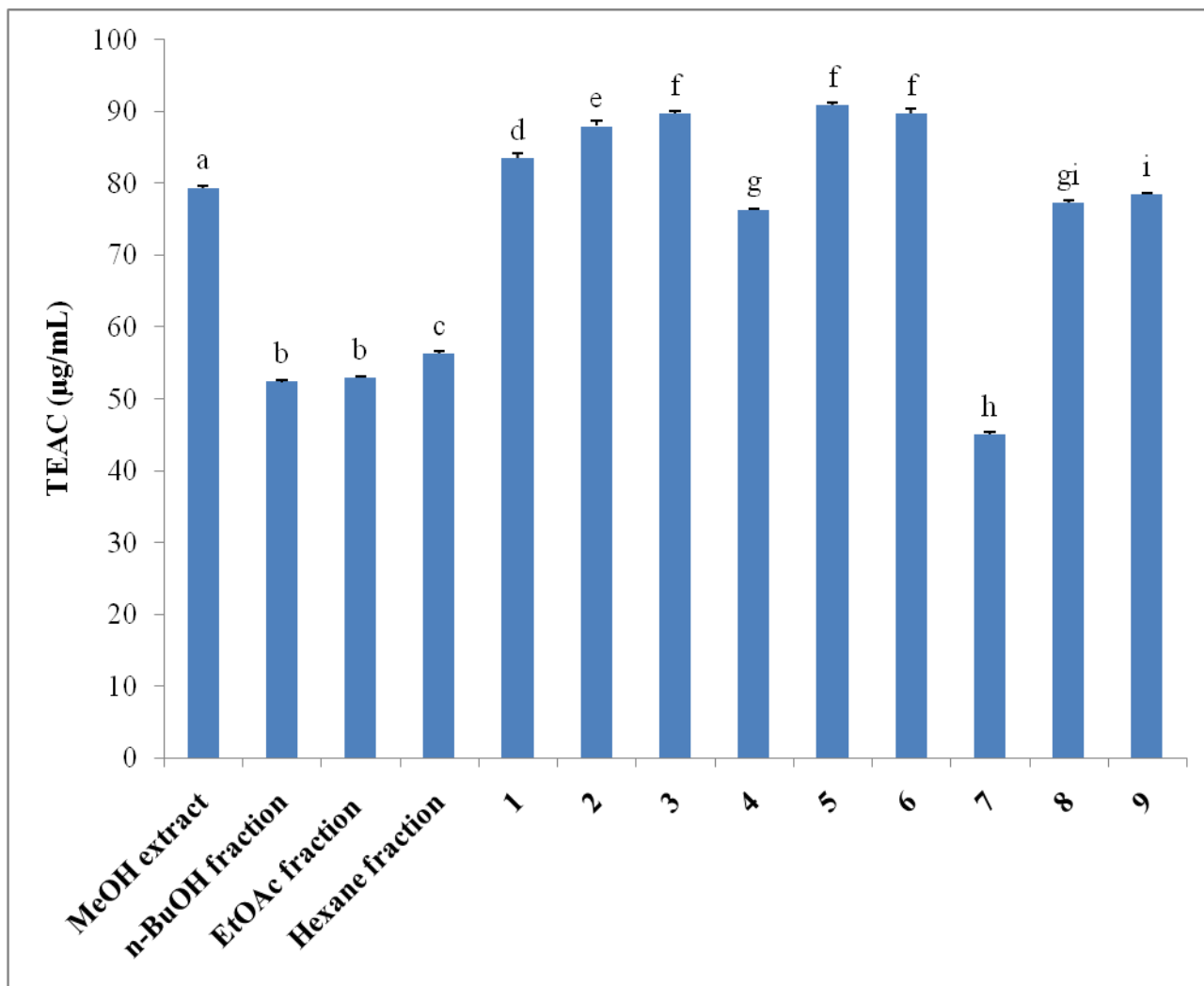
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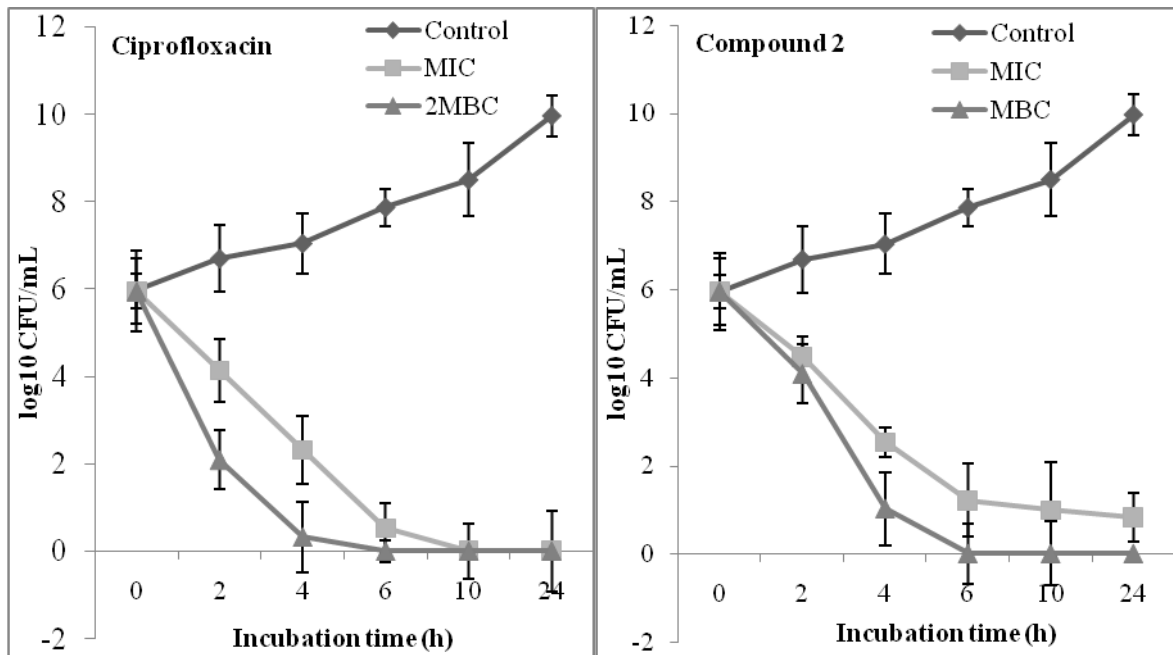
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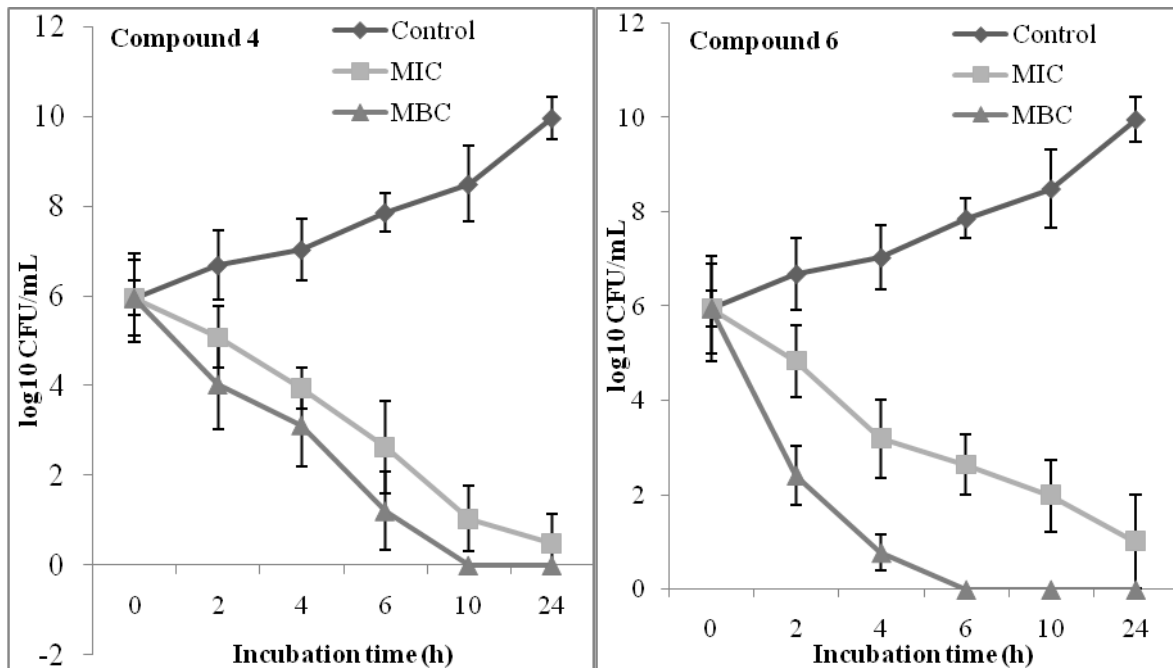
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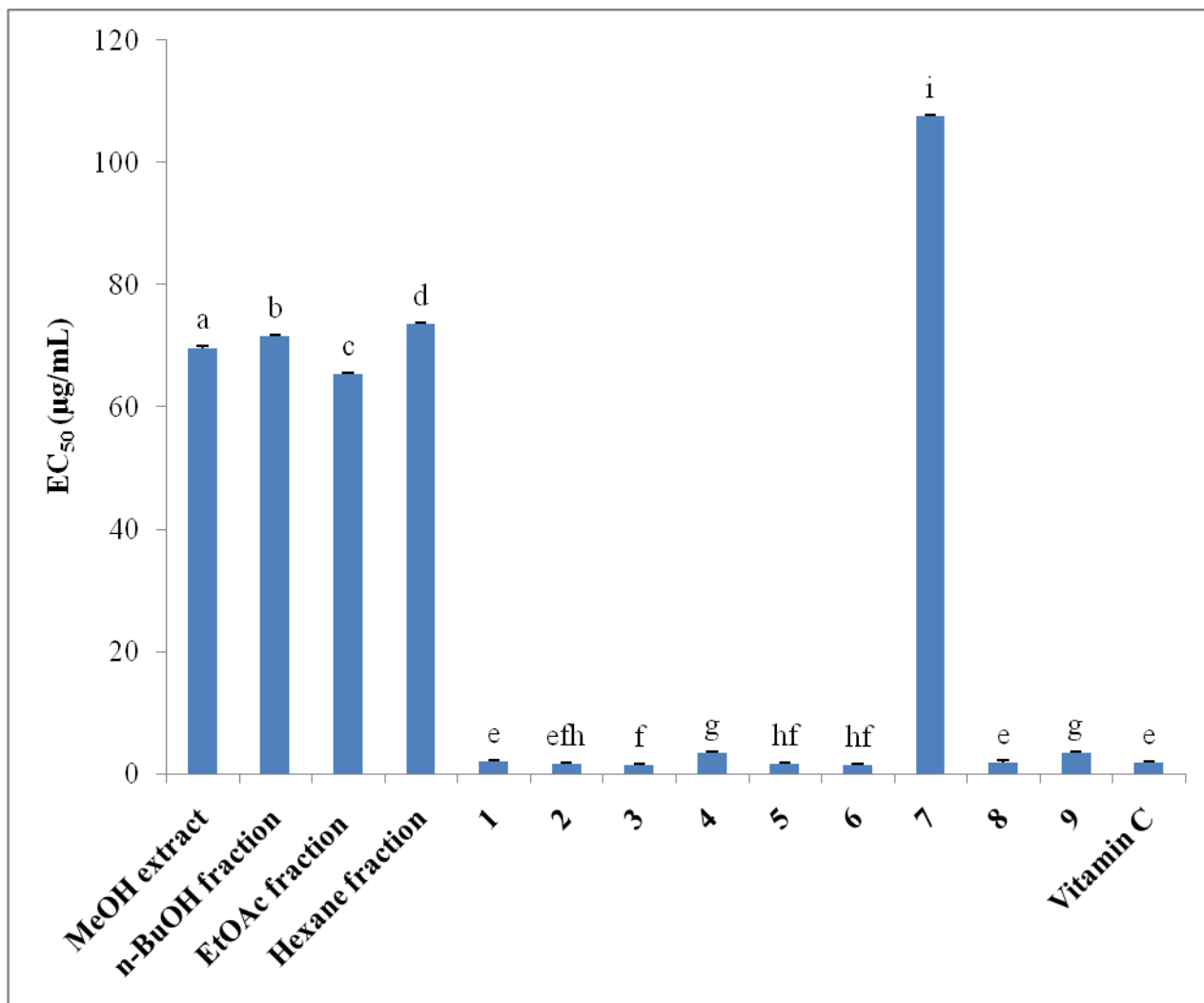and ciprofloxacin. Control: MHB medium with DMSO 1% + inoculums.

661

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

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