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1 **Antimicrobial activities of flavonoid glycosides from *Graptophyllum***
2 ***grandulosum* and their mechanism of antibacterial action**

3
4 Cyrille Ngoufack Tagousop¹, Jean-de-Dieu Tamokou^{2*}, Steve Endeguele Ekom², David
5 Ngnokam^{1**}, Laurence Voutquenne-Nazabadioko³

6
7 ¹Research Unit of Environmental and Applied Chemistry, Department of Chemistry, Faculty
8 of Science, University of Dschang, P.O. Box 67 Dschang, Cameroon.

9 ²Research Unit of Microbiology and Antimicrobial Substances, Department of Biochemistry,
10 Faculty of Science, University of Dschang, P.O. Box 67 Dschang, Cameroon.

11 ³*Groupe Isolement et Structure, Institut de Chimie Moléculaire de Reims (ICMR), CNRS*
12 *UMR 7312, Bat. 18 BP.1039, 51687 Reims cedex 2, France.*

13
14 **E-mail addresses for all authors:** Cyrille Ngoufack Tagousop -cyrilngoufack@yahoo.fr;
15 Jean-de-Dieu Tamokou -jtamokou@yahoo.fr; Steve Endeguele Ekom -
16 ekomsteve@yahoo.com; David Ngnokam -dngnokam@yahoo.fr; Laurence Voutquenne-
17 Nazabadioko -laurence.voutquenne@univ-reims.fr

18 **Correspondence**

19 *Doctor Jean-de-Dieu Tamokou, Research Unit of Microbiology and Antimicrobial
20 Substances, Department of Biochemistry, Faculty of Science, University of Dschang, PO.
21 Box 67 Dschang, Cameroon, Tel: +237 677 000 897. E-mail: jtamokou@yahoo.fr /
22 jean.tamokou@univ-dschang.org

24 **Doctor David Ngnokam, Research Unit of Environmental and Applied Chemistry,
25 Department of Chemistry, Faculty of Science, University of Dschang, PO. Box 67, Dschang,
26 Cameroon, Tel: +237 696 710 992. E-mail: dngnokam@yahoo.fr/ngnokam@univ-
27 dschang.org

28

29 **Abstract**

30 **Background:** The search for new antimicrobials should take into account drug resistance
31 phenomenon. Medicinal plants are known as sources of potent antimicrobial compounds
32 including flavonoids. The objective of this investigation was to evaluate the antimicrobial
33 activities of flavonoid glycosides from *Graptophyllum grandulosum*, as well as to determine
34 their mechanism of antibacterial action using lysis, leakage and osmotic stress assays.

35 **Methods:** The plant extracts were prepared by maceration in organic solvents. Column
36 chromatography of the *n*-butanol extract followed by purification of different fractions led to
37 the isolation of five flavonoid glycosides. The antimicrobial activities of extracts/compounds
38 were evaluated using the broth microdilution method. The bacteriolytic activity was evaluated
39 using the time-kill kinetic method. The effect of extracts on the red blood cells and bacterial
40 cell membrane was determined by spectrophotometric methods.

41 **Results:** Chrysoeriol-7-*O*- β -D-xyloside (1), luteolin-7-*O*- β -D-apiofuranosyl-(1 \rightarrow 2)- β -D-
42 xylopyranoside (2), chrysoeriol-7-*O*- β -D-apiofuranosyl-(1 \rightarrow 2)- β -D-xylopyranoside (3),
43 chrysoeriol-7-*O*- α -L-rhamnopyranosyl-(1 \rightarrow 6)- β -D-(4"-hydrogeno sulfate) glucopyranoside
44 (4) and isorhamnetin-3-*O*- α -L-rhamnopyranosyl-(1 \rightarrow 6)- β -D-glucopyranoside (5) were
45 isolated from *G. grandulosum* and showed different degrees of antimicrobial activities. Their
46 antibacterial activities against multi-drug-resistant *Vibrio cholerae* strains were in some cases
47 equal to, or higher than those of ciprofloxacin used as reference antibiotic. The antibacterial

48 activities of flavonoid glycosides and chloramphenicol increased under osmotic stress (5%
49 NaCl) whereas that of vancomycin decreased under this condition. *V. cholerae* suspension
50 treated with flavonoid glycosides, showed a significant increase in the optical density at 260
51 nm, suggesting that nucleic acids were lost through a damaged cytoplasmic membrane. A
52 decrease in the optical density of *V. cholerae* NB2 suspension treated with the isolated
53 compounds was observed, indicating the lysis of bacterial cells. The tested samples were non-
54 toxic to normal cells highlighting their good selectivity index.

55 **Conclusions:** The results of the present study indicate that the purified flavonoids from *G.*
56 *glandulosum* possess antimicrobial activities. Their mode of antibacterial activity is due to
57 cell lysis and disruption of the cytoplasmic membrane upon membrane permeability.

58 **Keywords:** *Graptophyllum glandulosum*; Acanthaceae; flavonoid glycosides; Antibacterial;
59 Antifungal; mode of action

60

61 **Background**

62 The development of resistance by microorganisms to existing antimicrobial agents has been
63 known for a long time. In several findings, the emergence of multidrug resistant strains of
64 *Vibrio cholerae* O1 and *Shigella flexneri* has been reported due to different genetic factors
65 including transfer of plasmids, integrons and allelic variation in the specific genes [1]. In
66 developing countries, fluoroquinolones, widely used for the treatment of many bacterial
67 diseases, including cholera and shigellosis, could contribute to the emergence of multidrug
68 resistance among potential enteric pathogens. Although significant progress has been made in
69 microbiological research and in the control of many diseases caused by infectious
70 microorganisms, recurrent epidemics due to drug-resistant microorganisms as well as the
71 appearance of new microbial pathogenic strains demand the discovery of new antibiotics. The
72 need for ecologically safe compounds as therapeutic agents against drug-resistant

73 microorganisms has driven many studies toward medicinal plants. Literature shows thousands
74 of plant species that have been tested *in vitro* against many fungal and bacterial strains, and a
75 good number of medicinal plant extracts and pure compounds have now been proven to be
76 active against fungi, Gram-positive and Gram-negative bacteria [2-5]. Medicinal plants
77 contain therapeutic amount of secondary metabolites including flavonoids. These are
78 polyphenolic and C₆-C₃-C₆ compounds in which the two C₆ groups are substituted benzene
79 rings, and the C₃ is an aliphatic chain which contains a pyran ring [6]. They occur as O- or C-
80 glycosides or in the free state as aglycones with hydroxyl or methoxyl groups [7]. The sugar
81 moiety is an important factor determining their bioavailability. Flavonoids may be divided
82 into seven types: flavones, flavonols, flavonones, flavanes, isoflavones, biflavones and
83 chalcones [8]. Flavonoids are well documented for their pharmacological effects, including
84 antimicrobial, anticancer, antiviral, antimutagenic and antiinflammatory activities [9-11].
85 Biological properties of flavonoids are linked to their ability to act as strong antioxidants and
86 free radical scavengers, to chelate metals, and to interact with enzymes, adenosine receptors
87 and biomembranes [7].

88 *Graptophyllum glandulosum* Turrill (Acanthaceae) is a shrub with 4-angled, nearly glabrous
89 branches, normal green leaves, and red-purple flowers 1 inch or more long. It is one of several
90 shrubs and trees of *Graptophyllum* that mainly grow in West and Central Africa but also in
91 the pacific regions [12]. This plant contains some important secondary metabolites such as
92 polyphenols, flavonoids and glycosides [13]. Leaves, roots and other parts of *G. glandulosum*,
93 are used in folk medicine in Cameroon to treat wounds, abscesses, skin diseases, respiratory
94 tract infections and diarrhea. Medical importance of this plant attracted us to explore its
95 antimicrobial properties. Although several ethnobotanical reports have emphasized the
96 pharmacological importance of this species for conditions that appear to be associated with
97 microbial infections, there is very limited literature concerned with the identification of the

98 antimicrobial compounds from this plant. However, a few reports on the *in vitro* antimicrobial
99 activity of plants have been published [14-15]. The objective of this investigation was to
100 evaluate the antimicrobial activities of flavonoid glycosides from *G. grandulosum*, as well as
101 to determine their mechanism of action using lysis, leakage, and osmotic stress assays.

102

103 **Methods**

104 **General experimental procedures**

105 **Melting point**

106 A Schorpp Gerätetechnik (Germany) apparatus was used to take the melting points of
107 different compounds.

108

109 **NMR analysis**

110 The 1D (^1H and ^{13}C -NMR) and 2D (COSY, NOESY, HSQC and HMBC) spectra were
111 performed in deuterated solvents (CD_3OD) on Bruker Avance III 600 spectrometer at 600
112 MHz/150 MHz. All chemical shifts (δ) are given in ppm units with reference to
113 tetramethylsilane (TMS) as internal standard and the coupling constants (J) are in Hz.

114 **Spectrometric analysis**

115 The mass spectra (HR-TOFESIMS) were carried out on Micromass Q-TOF micro instrument
116 (Manchester, UK). Samples were introduced by direct infusion in a solution of MeOH at a
117 rate of 5 $\mu\text{L}/\text{min}$.

118

119 **Chromatographic methods**

120 Silica gel 60 Merck, 70-230 mesh and sephadex LH-20 were used to perform column
121 chromatography while precoated silica gel 60 F₂₅₄ (Merck) plates, were used to perform thin
122 layer chromatography. The spots were visualized by an UV lamp multiband UV-254/365 nm
123 (ModelUVGL-58 Upland CA 91786, U.S.A) followed by spraying with 50% H₂SO₄ and
124 heating at 100 °C for 5 min.

125

126 **Plant material**

127 The aerial parts of *G. grandulosum* were harvested in a small village called Foto
128 situated in the Menoua Division, Western region of Cameroon) in November 2015. The Plant
129 was identified and authenticated by a Cameroonian Botanist (Mr. Fulbert Tadjouteu) at the
130 National Herbarium where a voucher specimen was archived (N° 65631/HNC).

131

132 **Extraction and isolation**

133 The extraction and isolation of compounds were done as previously described [13]. Briefly,
134 the aerial part of *G. grandulosum* was air-dried and powdered. The powder was macerated at
135 room temperature with MeOH to afford the MeOH extract. Part of this extract (235 g) was
136 suspended in water (300 mL) and successively partitioned with EtOAc and *n*-BuOH to yield
137 37 and 13 g of extracts, respectively. Column chromatography of the *n*-BuOH extract
138 followed by purification of different fractions led to the isolation of five compounds.

139

140 **Structural identification of the isolated compounds**

141 The structures of isolated compounds were determined after interpretation of their physical,
142 spectrometric and spectroscopic data summarized in this subsection.

143 Chrysoeriol-7-*O*- β -D-xyloside (**1**): yellow amorphous powder; molecular formula C₂₁H₂₀O₁₀;
144 ¹³C NMR (CD₃OD, 150 MHz) δ_C : 165.3 (C-2), 104.3 (C-3), 184.1 (C-4), 161.7 (C-5), 99.6
145 (C-6), 163.1 (C-7), 95.8 (C-8), 158.5 (C-9), 107.0 (C-10), 123.1 (C-1'), 110.5 (C-2'), 148.2
146 (C-3'), 151.0 (C-4'), 116.7 (C-5'), 121.9 (C-6'), 55.2 (C-7') for aglycone; 100.9 (C-1''), 74.4
147 (C-2''), 77.3 (C-3''), 70.7 (C-4''), 66.9 (C-5'') for sugar moiety. ¹H NMR data (CD₃OD, 600
148 MHz) δ_H : 6.62 (1H, s, H-3), 6.38 (1H, d, *J* = 2.1 Hz, H-6), 6.71 (1H, d, *J* = 2.1 Hz, H-8), 7.44
149 (1H, d, *J* = 2.1 Hz, H-2'), 6.84 (1H, d, *J* = 8.4 Hz, H-5'), 7.47 (1H, dd, *J* = 8.4 and 2.1 Hz, H-
150 6'), 3.87 (3H, s, H-7') for aglycone; 4.95 (1H, d, *J* = 7.1 Hz, H-1''), 3.37 (1H, m, H-2''), 3.36
151 (1H, m, H-3''), 3.49 (1H, m, H-4''), 3.38 (1H, m, H-5''a), 3.87 (1H, m, H-5''b) for sugar
152 moiety.

153 Luteolin-7-*O*- β -D-apiofuranosyl-(1 \rightarrow 2)- β -D-xylopyranoside (**2**): yellow powder; molecular
154 formula C₂₅H₂₆O₁₄. m.p. = 203 °C. ¹³C NMR data (CD₃OD, 150 MHz) δ_C : 165.3 (C-2), 103.5
155 (C-3), 182.5 (C-4), 162.9 (C-5), 99.7 (C-6), 163.1 (C-7), 94.9 (C-8), 157.4 (C-9), 105.5 (C-
156 10), 121.7 (C-1'), 114.2 (C-2'), 146.4 (C-3'), 150.5 (C-4'), 116.6 (C-5'), 119.7 (C-6') for
157 aglycone; 99.0 (C-1''), 76.0 (C-2''), 77.0 (C-3''), 70.0 (C-4''), 66.1 (C-5''), 109.3 (C-1'''), 76.5
158 (C-2'''), 79.7 (C-3'''), 64.5 (C-4'''), 74.4 (C-5''') for sugar moiety. ¹H NMR data (CD₃OD, 600
159 MHz) δ_H : 6.75 (1H, s, H-3), 6.40 (1H, d, *J* = 2.1 Hz, H-6), 6.75 (1H, d, *J* = 2.1 Hz, H-8), 7.44
160 (1H, d, *J* = 2.1 Hz, H-2'), 6.90 (1H, d, *J* = 8.4 Hz, H-5'), 7.47 (1H, dd, *J* = 8.4 and 2.1 Hz, H-
161 6') for aglycone; 5.18 (1H, d, *J* = 7.1 Hz, H-1''), 3.52 (1H, dd, *J* = 9.0 and 7.1 Hz, H-2''), 3.43
162 (1H, m, H-3''), 3.41 (1H, m, H-4''), 3.78 (1H, dd, *J* = 9.7 and 3.4 Hz, H-5''a), 3.42 (1H, dd, *J*
163 = 9.7 and 3.4 Hz, H-5''b), 5.34 (1H, d, *J* = 1.3 Hz, H-1'''), 3.75 (1H, m, H-2'''), 3.30 (2H, d, *J*
164 = 3.4 Hz, H-4'''), 3.88 (1H, d, *J* = 9.3 Hz, H-5'''a), 3.65 (1H, d, *J* = 9.3 Hz, H-5'''b) for sugar
165 moiety.

166 Chrysoeriol-7-*O*- β -D-apiofuranosyl-(1 \rightarrow 2)- β -D-xylopyranoside (**3**): yellow powder;
167 molecular formula C₂₆H₂₈O₄; melting point = 181.8 °C. ¹³C NMR (CD₃OD, 150 MHz) δ_C :

168 166.6 (C-2), 104.5 (C-3), 184.0 (C-4), 162.9 (C-5), 100.9 (C-6), 164.4 (C-7), 95.9 (C-8),
169 158.9 (C-9), 107.0 (C-10), 123.4 (C-1'), 110.4 (C-2'), 149.5 (C-3'), 152.3 (C-4'), 116.7 (C-5'),
170 121.9 (C-6'), 56.6 (C-7') for aglycone; 100.6 (C-1''), 78.6 (C-2''), 77.9 (C-3''), 70.9 (C-4''),
171 66.9 (C-5''), 110.0 (C-1'''), 78.1 (C-2'''), 80.7 (C-3'''), 65.8 (C-4'''), 75.4 (C-5''') for sugar
172 moiety. ¹H NMR data (CD₃OD, 600 MHz) δ_H : 6.70 (1H, s, H-3), 6.45 (1H, d, $J = 2.1$ Hz, H-
173 6), 6.77 (1H, d, $J = 2.1$ Hz, H-8), 7.52 (1H, d, $J = 2.1$ Hz, H-2'), 6.95 (1H, d, $J = 8.4$ Hz, H-
174 5'), 7.56(1H, dd, $J = 8.4$ and 2.1 Hz, H-6'), 3.98 (3H, s, H-7') for aglycone; 5.16 (1H, d, $J =$
175 7.1 Hz, H-1''), 3.68 (1H, dd, $J = 9.0$ and 7.1 , H-2''), 3.63 (1H, m, H-3''), 3.62 (1H, m, H-4''),
176 3.98 (1H, m, H-5''a), 3.48 (1H, t, $J = 9.6$, H-5''b), 5.46 (1H, d, $J = 1.7$ Hz, H-1'''), 3.98 (1H,
177 m, H-2'''), 3.56 (2H, brs, H-4'''), 4.05 (1H, d, $J = 9.4$, H-5'''a), 3.84 (1H, d, $J = 9.4$, H-5'''b) for
178 sugar moiety.

179 Chrysoeriol-7-*O*- α -L-rhamnopyranosyl-(1 \rightarrow 6)- β -D-(4''-hydrogenosulfate) glucopyranoside
180 (**4**): yellow amorphous powder; molecular formula C₂₈H₃₁NaO₁₈S. ¹³C NMR data (CD₃OD,
181 150 MHz) δ_C : 166.8 (C-2), 104.5 (C-3), 184.1 (C-4), 163.1 (C-5), 101.1 (C-6), 164.0 (C-7),
182 96.2 (C-8), 159.0 (C-9), 107.2 (C-10), 123.6 (C-1'), 110.8 (C-2'), 149.6 (C-3'), 152.3 (C-4'),
183 116.9 (C-5'), 122.0 (C-6'), 56.7 (C-7') for aglycone; 100.9 (C-1''), 74.5 (C-2''), 76.8 (C-3''),
184 77.5 (C-4''), 75.3 (C-5''), 67.1 (C-6''), 102.4 (C-1'''), 71.9 (C-2'''), 72.3 (C-3'''), 74.2 (C-4'''),
185 69.8 (C-5'''), 17.9 (C-6''') for sugar moiety. ¹H NMR data (CD₃OD, 600 MHz) δ_H : 6.73 (1H,
186 s, H-3), 6.56 (1H, d, $J = 2.1$ Hz, H-6), 6.84 (1H, d, $J = 2.1$ Hz, H-8), 7.55 (1H, d, $J = 2.1$ Hz,
187 H-2'), 6.98 (1H, d, $J = 8.4$ Hz, H-5'), 7.59 (1H, dd, $J = 8.4$ and 2.1 Hz, H-6'), 3.99 (3H, s, H-
188 7') for aglycone; 5.15 (1H, d, $J = 7.8$ Hz, H-1''), 3.61 (1H, dd, $J = 9.1$ and 7.8 Hz, H-2''), 3.84
189 (1H, t, $J = 9.1$ Hz, H-3''), 4.32 (1H, dd, $J = 9.9$ and 9.1 Hz, H-4''), 3.89 (1H, m, H-5''), 4.10
190 (1H, m, H-6''a), 3.68 (1H, m, H-6''b), 4.75 (1H, d, $J = 1.3$ Hz, H-1'''), 3.95 (1H, dd, $J = 3.4$
191 and 1.3 , H-2'''), 3.72 (1H, dd, $J = 9.5$ and 3.4 Hz, H-3'''), 3.32 (1H, t, $J = 9.5$ Hz, H-4'''), 3.62
192 (1H, m, H-5'''), 1.21 (3H, d, $J = 6.2$ Hz, H-6''') for sugar moiety.

193 Isorhamnetin-3-*O*- α -L-rhamnopyranosyl-(1 \rightarrow 6)- β -D-glucopyranoside (**5**): yellow amorphous
194 powder; molecular formula C₂₈H₃₂O₁₅. ¹³C NMR data (CD₃OD, 150 MHz) δ_C : 159.8 (C-2),
195 135.7 (C-3), 179.8 (C-4), 162.4 (C-5), 104.3 (C-6), 160.1 (C-7), 100.2 (C-8), 157.5 (C-9),
196 108.6 (C-10), 123.0 (C-1'), 114.5 (C-2'), 148.4 (C-3'), 151.1 (C-4'), 116.2 (C-5'), 124.4 (C-6'),
197 56.7 (C-7') for aglycone; 104.0 (C-1''), 75.9 (C-2''), 78.1 (C-3''), 71.8 (C-4''), 77.4 (C-5''),
198 68.7 (C-6''), 102.6 (C-1'''), 72.1 (C-2'''), 72.3 (C-3'''), 73.8 (C-4'''), 69.8 (C-5'''), 18.0 (C-6''')
199 for sugar moiety. ¹H NMR data (CD₃OD, 600 MHz) δ_H : 6.71 (1H, d, *J* = 2.2 Hz, H-6), 7.09
200 (1H, d, *J* = 2.2 Hz, H-8), 7.91 (1H, d, *J* = 2.2 Hz, H-2'), 6.95 (1H, d, *J* = 8.5 Hz, H-5'), 7.72
201 (1H, dd, *J* = 8.5 and 2.2 Hz, H-6'), 3.98 (3H, s, H-7') for aglycone; 5.30 (1H, d, *J* = 7.6 Hz, H-
202 1''), 3.49 (1H, dd, *J* = 9.1 and 7.6 Hz, H-2''), 3.45 (1H, t, *J* = 9.1 Hz, H-3''), 3.23 (1H, t, *J* =
203 9.1 Hz, H-4''), 4.40 (1H, m, H-5''), 3.84 (1H, dd, *J* = 12.1 and 1.9 Hz, H-6''a), 3.42 (1H, m, H-
204 6''b), 4.53 (1H, d, *J* = 1.3 Hz, H-1'''), 3.58 (1H, dd, *J* = 3.4 and 1.3, H-2'''), 3.47 (1H, dd, *J* =
205 9.5 and 3.4 Hz, H-3'''), 3.25 (1H, t, *J* = 9.5 Hz, H-4'''), 3.41 (1H, m, H-5'''), 1.10 (3H, d, *J* =
206 6.2 Hz, H-6''') for sugar moiety.

207

208 **Antimicrobial assay**

209 **Microorganisms**

210 The microorganisms used in this study were consisted of five bacterial strains namely
211 *Staphylococcus aureus* ATCC 25923, *Vibrio cholerae* NB2, PC2, SG24 (1) and CO6 [16].
212 Also included were two fungi *Candida albicans* ATCC 9002 and *Cryptococcus neoformans*
213 IP95026. These bacteria and yeasts were obtained from our local stocks.

214

215 **Determination of minimum inhibitory concentration and minimum microbicidal** 216 **concentration**

217 The minimum inhibitory concentration (MIC) values were determined using the broth micro-
218 dilution method as described earlier [17]. The MIC values were defined as the lowest sample
219 concentration that prevented the change in color indicating a complete inhibition of microbial
220 growth. The lowest concentrations that yielded no growth after the subculturing were taken as
221 the minimum microbicidal concentration (MMC) values [18]. Ciprofloxacin (Sigma-Aldrich,
222 Steinheim, Germany) and amphotericin B (Merck, Darmstadt, Germany) were used as
223 positive controls for bacteria and yeast respectively.

224

225 **Study on mode of action**

226 **MIC and MBC changes under osmotic stress condition**

227 Osmotic stress was induced by adding 5% NaCl (w/v) to MHB. The MHB supplemented with
228 5% NaCl was then sterilized and used for the determination of a new MIC and MBC values of
229 the samples as previously described [17]. The incubation time was increased from 24 to 48 h
230 at 37 °C.

231

232 **Effect of isolated compounds on cell membrane**

233 The alteration of cell membrane of *V. cholerae* NB2 was evaluated by measuring the optical
234 densities at 260 nm of the bacterial suspensions in the presence and absence of compounds **1 -**
235 **5** using the method described by Carson et al. [19]. For this purpose, the compounds were
236 tested at their MIC using 1 mL of the bacterial suspension (approximately 10⁸ CFU/mL). The
237 mixture was then incubated at 37 °C at different time intervals (0: immediately after addition
238 of the compound; 15; 30; 60 min), 50 µL of the mixture was taken and mixed with 1.95 mL of
239 Phosphate Buffered Saline (PBS Buffer). The absorbance was measured on the
240 spectrophotometer at 260 nm against the blank (PBS). For the negative control, 1 mL of
241 bacterial suspension was incubated at 37 °C and 50 µL of the suspension was removed at the

242 end of the various incubation times and mixed with 1.95 mL of Buffer. The optical densities
243 were read in the same way.

244

245 **Bacteriolytic assay**

246 The bacteriolytic activities of the isolated compounds were determined using the time-
247 kill kinetic method as previously described [20] with slight modifications. Full growth of *V.*
248 *cholerae* NB2 in MHB was diluted 100 times and incubated at 37 °C to produce an OD₆₀₀ of
249 0.8 as starting inoculum. Sample solutions were added to the starting bacterial suspension to
250 give a final concentration of 2 × MIC and incubated at 37 °C with shaking, then 100 µL was
251 removed from each tube at 0, 15, 30, 60, and 120 min and the optical density measured at 600
252 nm. Vancomycin and tetracycline were used as positive controls and the tubes without
253 isolated compounds served as negative controls.

254

255 **Hemolytic assay**

256 Whole blood (10 mL) from albino rats was collected by cardiac puncture in EDTA tubes. The
257 study was conducted according to the ethical guidelines of the Committee for Control and
258 Supervision of Experiments on Animals (Registration no. 173/CPCSEA, dated 28 January,
259 2000), Government of India, on the use of animals for scientific research. Erythrocytes were
260 harvested by centrifugation at room temperature for 10 min at 1,000 x g and were washed
261 three times in PBS buffer [21]. The cytotoxicity was evaluated as previously described [21].

262

263 **Statistical analysis**

264 Data were analyzed by one-way analysis of variance followed by Waller-Duncan *post*
265 *hoc* test. The experimental results were expressed as the mean ± Standard Deviation (SD).

266 Differences between groups were considered significant when $p < 0.05$. All analyses were
267 performed using the Statistical Package for Social Sciences (SPSS, version 12.0) software.

268

269 **Results**

270 **Chemical analysis**

271 The structures of five known flavonoid glycosides isolated from the *n*-BuOH fraction of
272 leaves of *G. grandulosum* (Figure 1) were determined using spectroscopic analysis and NMR
273 spectra in conjunction with 2D experiments (COSY, NOESY, HSQC and HMBC). Direct
274 comparison with published information led to the identification of chrysoeriol-7-*O*- β -D-
275 xyloside **1** [22], luteolin-7-*O*- β -D-apiofuranosyl-(1 \rightarrow 2)- β -D-xylopyranoside **2** [23],
276 chrysoeriol-7-*O*- β -D-apiofuranosyl-(1 \rightarrow 2)- β -D-xylopyranoside **3** [13], chrysoeriol-7-*O*- α -L-
277 rhamnopyranosyl-(1 \rightarrow 6)- β -D-(4''-hydrogeno sulfate) glucopyranoside **4** [13] and
278 isorhamnetin-3-*O*- α -L-rhamnopyranosyl-(1 \rightarrow 6)- β -D-glucopyranoside **5** [24].

279

280 **Antimicrobial activity**

281 The *in vitro* activities of MeOH, *n*-BuOH and EtOAc extracts as well as their isolated
282 compounds against pathogenic bacteria and fungi are presented in Table 1. The test samples
283 demonstrated varying degrees of inhibitory activities against the bacterial and fungal strains.
284 Fungal strains were generally more susceptible to the effects of the compounds, but less
285 susceptible to extracts. The EtOAc and *n*-BuOH extracts were active against *C. albicans* and
286 *C. neoformans* which were not susceptible to the MeOH extract. The MIC values obtained
287 with the EtOAc and *n*-BuOH extracts were smaller than those obtained with the MeOH
288 extract. These observations suggest that the fractionation of the MeOH extract enhanced its
289 antimicrobial activity. The lowest MIC values were recorded on *S. aureus*; suggesting that
290 this microorganism was the most susceptible to all the test samples. The EtOAc extract

291 showed the highest antimicrobial activity when compared with the MeOH and *n*-BuOH
292 extracts.

293 The antimicrobial activities of the isolated compounds from *G. glandulosum* were as follows:
294 compound **4** > compound **1** > compound **2** > compound **5** > compound **3**. The lowest MIC
295 value of 4 µg/mL was recorded on *C. neoformans* with compound **4** and on *S. aureus* with
296 compounds **1**, **2** and **4** whereas the lowest MMC value was obtained on *S. aureus* with
297 compound **4**. However, the highest MIC value for compounds (64 µg/mL) was recorded with
298 compound **3** against *V. cholerae* CO6, and with compounds **2** and **5** against *C. albicans*, while
299 the highest MBC value of 128 µg/mL was obtained on *V. cholerae* CO6 with compound **3** and
300 on *C. albicans* with compound **5**.

301

302 **Antibacterial activity of flavonoid glycosides under osmotic stress condition**

303 The MIC values of flavonoid glycosides against Gram-negative and Gram-positive bacteria
304 are reported in Table 2. The results clearly showed that the MIC values of flavonoid
305 glycosides obtained under osmotic stress (in the presence of 5% NaCl) are smaller than those
306 obtained under normal conditions (0% NaCl). This result suggests an increase in the activity
307 of purified flavonoid glycosides under osmotic stress. As demonstrated under normal
308 condition, compound **4** was still the most effective under osmotic stress, followed in
309 decreasing order by compounds **1** and **2**. The MIC values of chloramphenicol determined
310 under osmotic stress condition were smaller than those determined under normal conditions.
311 However, all the MIC values of vancomycin determined under osmotic stress were higher
312 than those determined under normal conditions. Table 1 further shows that under osmotic
313 stress, the antibacterial activities of compounds **1**, **2** and **4** against *V. choroae* SG24 (1), *V.*
314 *chorae* CO6, *V. choroae* NB2 and *V. choroae* PC2 were higher than that of vancomycin.

315 **Effect of flavonoid glycosides on cell membrane**

316 The effect of flavonoid glycosides of *G. glandulosum* was evaluated in terms of leakage of
317 UV 260 absorbing material through the bacterial cell membrane (Figure 2). After treatment
318 with flavonoid glycosides at MIC values of compounds **1**, **2** and **4**, the OD₂₆₀ values of
319 filtrates of all test strains increased and most of the leakage occurred during the initial period
320 (≤ 15 min), followed by a slight increase with prolonged incubation period. At the same time,
321 the OD₂₆₀ of the control without compound was not changed. These results suggest that
322 flavonoid glycosides from *G. glandulosum* damage the cytoplasmic membrane and cause loss
323 of intracellular components. The highest values of OD₂₆₀ were recorded with compound **4** for
324 all the *V. cholerae* strains, whereas the least OD₂₆₀ values were noticed with compound **2**,
325 indicating that compound **4** released the highest amounts of nucleic acids followed in
326 decreasing order by compound **1**, then **2**.

327 **Bacteriolytic effect of compounds 1, 2 and 4**

328 The result on the leakage of 260 nm absorbing material was consistent with that of
329 bacteriolysis (Figure 3). This result showed a decrease in the optical density of suspension
330 treated with compounds **1**, **2** and **4**. After 120 min, compounds **1**, **2** and **4** induced a decline in
331 cell turbidity of 93.20, 94.36 and 95.16%, respectively in bacteria suspension compared to
332 time 0, indicating the lysis of bacterial cells.

333 **Haemolytic activity**

334 The haemolytic activities of extracts and compounds **1** – **5** against red blood cells (RBCs)
335 were investigated using Triton X-100 as a positive control. The positive control showed about
336 100% lysis, whereas the phosphate buffer saline (PBS) showed no lysis of RBCs.
337 Interestingly, none of the tested samples showed haemolytic activities against RBCs at
338 concentrations up to 256 and 2048 $\mu\text{g/mL}$ for isolated compounds and extracts respectively

339 (results not shown). This finding highlights the fact that the observed biological efficacy was
340 not due to haemolysis.

341 **Discussion**

342 The antimicrobial activity of a plant extract is considered to be highly active if the MIC < 100
343 µg/mL; significantly active when $100 \leq \text{MIC} \leq 512$ µg/mL; moderately active when $512 <$
344 $\text{MIC} \leq 2048$ µg/mL; weakly active if MIC > 2048 µg/mL and not active when MIC > 10 000
345 µg/mL [25]. Hence, the EtOAc extract of *G. glandulosum* was highly active (MIC < 100
346 µg/mL) against *V. cholerae* SG24 (1), *V. cholerae* NB2, *V. cholerae* PC2 and *S. aureus*;
347 significantly active ($100 \leq \text{MIC} \leq 512$ µg/mL) against *V. cholerae* CO6 and *C. neoformans*;
348 moderately active ($512 < \text{MIC} \leq 2048$ µg/mL) on *C. albicans*. The MeOH and *n*-BuOH
349 extracts were significantly active against the test bacterial species; weakly and moderately
350 active against the yeast cells respectively.

351 In this study, we also investigated if the mode of action of flavonoid compounds is
352 bactericidal or bacteriostatic. The results of the MMC values were fourfold lesser than their
353 corresponding MIC values. This observation suggests that the actions of extracts from *G.*
354 *glandulosum* and their isolated flavonoid glycosides were bactericidal [11].

355 The antibacterial activities of flavonoid glycosides were in some cases equal to, or higher than
356 those of ciprofloxacin used as reference antibiotic, suggesting that they might be effective
357 antibiotics against these pathogenic bacteria. Taking into account the medical importance of
358 the test microbial species, the result can be considered as promising for the development of
359 new antimicrobial drugs. The antimicrobial activities of purified flavonoids corroborated with
360 those of early reports against bacteria and fungi [5,11,26-28]. The antibacterial activity of the
361 samples against *V. cholerae* and *S. flexneri* are particularly noteworthy since these strains

362 were MDR clinical isolates which were resistant to commonly used drugs such as ampicillin,
363 streptomycin, nalidixic acid, furazolidone and co-trimoxazole [16,29-30].

364 Antimicrobial cutoff points have been defined in the literature to enable the understanding of
365 the effectiveness of pure compounds as follows: highly active: MIC below 1 $\mu\text{g/mL}$ (or 2.5
366 μM), significantly active: $1 \leq \text{MIC} \leq 10 \mu\text{g/mL}$ (or $2.5 \leq \text{MIC} < 25 \mu\text{M}$), moderately active:
367 $10 < \text{MIC} \leq 100 \mu\text{g/mL}$ (or $25 < \text{MIC} \leq 250 \mu\text{M}$), weakly active: $100 < \text{MIC} \leq 1000 \mu\text{g/mL}$
368 (or $250 < \text{MIC} \leq 2500 \mu\text{M}$ and not active: $\text{MIC} > 1000 \mu\text{g/mL}$ (or $> 2500 \mu\text{M}$) [25]. Based on
369 this, the antimicrobial activities of all the tested flavonoid glycosides could be considered as
370 significant or moderate against the specific microorganisms.

371 The antimicrobial activities of the isolated compounds from *G. glandulosum* were in this
372 order: compound **4** > compound **1** > compound **2** > compound **5** > compound **3**. Very little is
373 known about the structure–function relationships of natural antimicrobials, but it seems that
374 different substituent groups within the compounds had a great influence on their biophysical
375 and biological properties [31]. Structural features such as the presence of an aromatic ring, the
376 sugar moiety or the numbers of hydroxyl and methoxyl groups can significantly change
377 membrane permeability and subsequent affinity to external and internal binding sites in the
378 bacteria, thus influencing the compound's antimicrobial properties [32].

379 The antibacterial activities of flavonoid glycosides and chloramphenicol increased under
380 osmotic stress (5% NaCl) whereas that of vancomycin decreased under this condition. The
381 results were supported by the observation that certain bacterial strains (*E. coli*, *S. aureus*, *P.*
382 *aeruginosa*) can survive under osmotic stress conditions [33]. At low water activity, lipid
383 composition of bacterial cell membrane was changed [34]. This incident might lead to
384 occurrence of more antibacterial binding site on cell membrane of bacteria and cause less
385 resistance to antibacterial substance. Therefore, the presence of the salt triggered changes in

386 the membrane lipid composition. This is possible to increase the antibacterial activity of
387 flavonoid glycosides and chloramphenicol. However, the mechanisms that make bacteria
388 more sensitive to certain antibiotics under osmotic stress conditions are still unknown. The
389 results of vancomycin activity are in agreement with those of McMahon and coworkers [35]
390 who demonstrated a decrease in the activity of amikacin, ceftriaxone and trimethoprim against
391 *E. coli* and *S. aureus* under osmotic stress conditions.

392 Marked leakage of cytoplasmic material is considered indicative of gross and irreversible
393 damaged to the cytoplasmic membrane. Many antibacterial compounds that act on the
394 bacterial cytoplasmic membrane induce the loss of 260 nm-absorbing materials (nucleic
395 acids) including chlorohexidine, hexachlorophene, phenetyl alcohol, tetracycline, polymixin,
396 α -pinene, and lemon grass oil [19]. The *V. cholerae* suspension treated with flavonoid
397 glycosides, showed a significant increase in the optical density at 260 nm, suggesting that
398 nucleic acids were lost through a damaged cytoplasmic membrane.

399 Our observations confirm that the antimicrobial activity of flavonoid glycosides results from
400 their ability to disrupt the permeability barrier of microbial membrane structures. This mode
401 of action is similar to that of other broad-spectrum, membrane-active disinfectants and
402 preservatives, such as phenol derivatives, chlorohexidine and para benzoic acid derivatives
403 [36]. Furthermore, Devi and Kapila [37], reported the antibacterial mechanism as disruption
404 of plasma membrane by the phytochemicals in the extracts of Indian liverworts.

405 The fact that flavonoids-induced damage to cell membrane structure accompanied by the
406 decline in the absorbance of bacterial cell suspension treated with compounds has confirmed
407 it as the most likely cause of cell death. Our result is supported by the observation that other
408 flavonoid compounds such as epigallocatechin gallate and galangin induced 3-log reduction
409 or more in viable counts of *S. aureus* [38,39].

410 **Conclusions**

411 The results of the present study indicate that the purified flavonoid glycosides from *G.*
412 *glandulosum* possess antimicrobial activities. Their mode of antibacterial activity is due to
413 cell lysis and disruption of the cytoplasmic membrane by action upon the membrane
414 permeability leading to leakage of cellular components and eventually cell death. This will
415 lead to improve antimicrobial formulations and to ensure the prevention of the emergence of
416 microbial resistance. However, the possibility remains that sites of action other than the
417 cytoplasmic membrane exist. Further work is required to expatiate fully the mechanisms
418 involved.

419 **Abbreviations**

420 ¹³C-NMR: Carbon Thirteen Nuclear Magnetic Resonance; ¹H NMR: Proton Nuclear Magnetic
421 Resonance; ²D NMR: Two-dimension Nuclear Magnetic Resonance; ATCC: American Type
422 Culture Collection; CC: Column Chromatography; COSY: Correlation Spectroscopy; DMSO:
423 Dimethylsulfoxide; EtOAc: Ethyl acetate; HMBC: Heteronuclear Multiple Bond
424 Connectivities; HNC: *Herbier National du Cameroun*; HR-EI-MS: High Resolution Electron
425 Impact Mass Spectrometry; HR-TOFESIMS: High-Resolution Time of Flight Electrospray
426 Ionization Mass Spectrometry; HSQC: The Heteronuclear Single Quantum Coherence; IR :
427 Infra-red; MBC: Minimum bactericidal concentration; MDR: Multi-Drug-Resistant; MeOH:
428 Methanol; MHA: Mueller Hinton agar; MHB: Mueller Hinton broth; MIC: Minimum
429 inhibitory concentration; MMC: Minimum Microbicidal Concentration; NA: Nutrient agar; *n*-
430 BuOH: *n*-Butanol; NMR: Nuclear Magnetic Resonance; R_f: Retention factor; TLC: Thin
431 Layer Chromatography; TMS: Tetramethylsilane; TOF-ESIMS: Time of Flight Electrospray
432 Ionization Mass Spectrometry; UV: Ultra-violet

433 **Declarations**

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438 **Availability of data and materials**

439 The datasets used and/or analyzed during the current study are available from the
440 corresponding author on reasonable request.

441 **Authors' Contributions**

442 CNT and SEE contributed to the data collection and analysis. JDT designated the study, did
443 the biological assays and helped in manuscript writing and editing. JDT, DN and LVN
444 supervised and revised the manuscript critically for important intellectual content. All authors
445 read and agreed on the final version of the manuscript.

446 **Ethics approval and consent to participate**

447 Not applicable.

448 **Competing interests**

449 The Authors declare that there are no known competing interests associated with this work.

450 **Authors' Information**

451 ¹Research Unit of Environmental and Applied Chemistry, Department of Chemistry, Faculty
452 of Science, University of Dschang, P.O. Box 67 Dschang, Cameroon.

453 ²Research Unit of Microbiology and Antimicrobial Substances, Department of Biochemistry,
454 Faculty of Science, University of Dschang, P.O. Box 67 Dschang, Cameroon.

455 ³Groupe Isolement et Structure, Institut de Chimie Moléculaire de Reims (ICMR), CNRS
456 UMR 7312, Bat. 18 BP.1039, 51687 Reims cedex 2, France.

457 **Consent for publication**

458 Not applicable.

459

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- 569

570 **Figure Legends**

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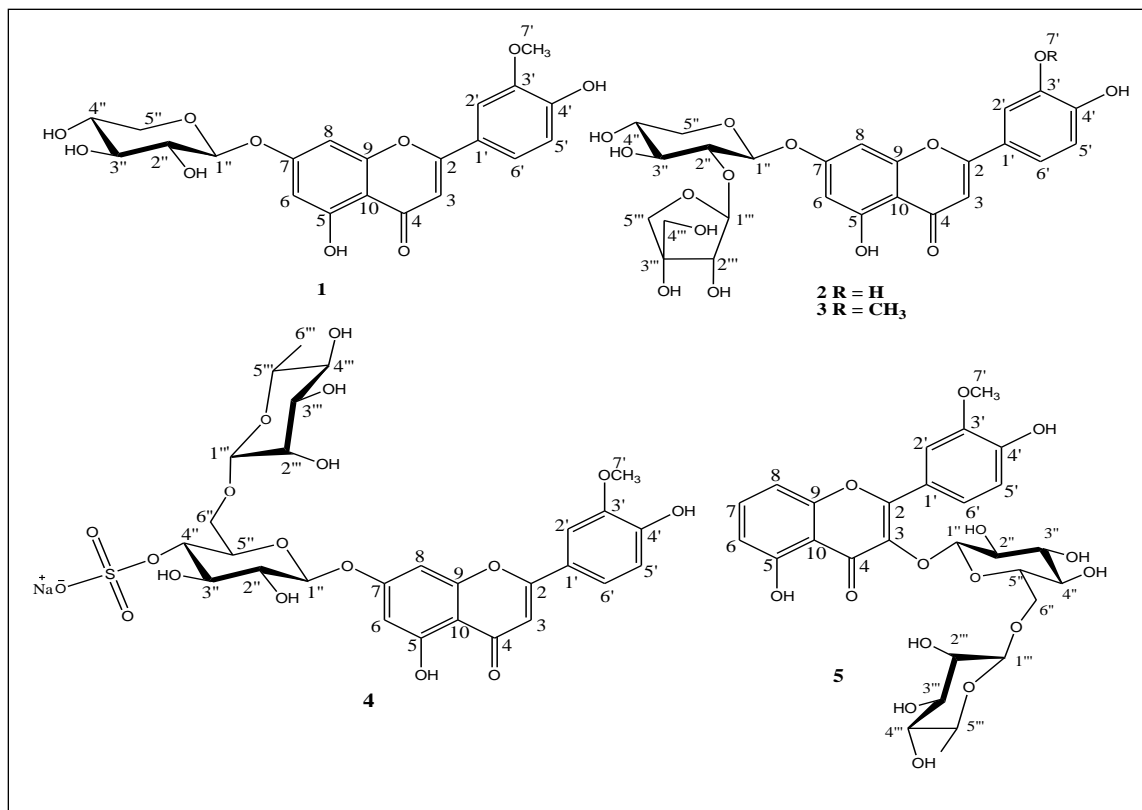
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584 **Fig. 1** Chemical structures of flavonoids (**1-5**) isolated from *n*-BuOH extract of aerial parts of

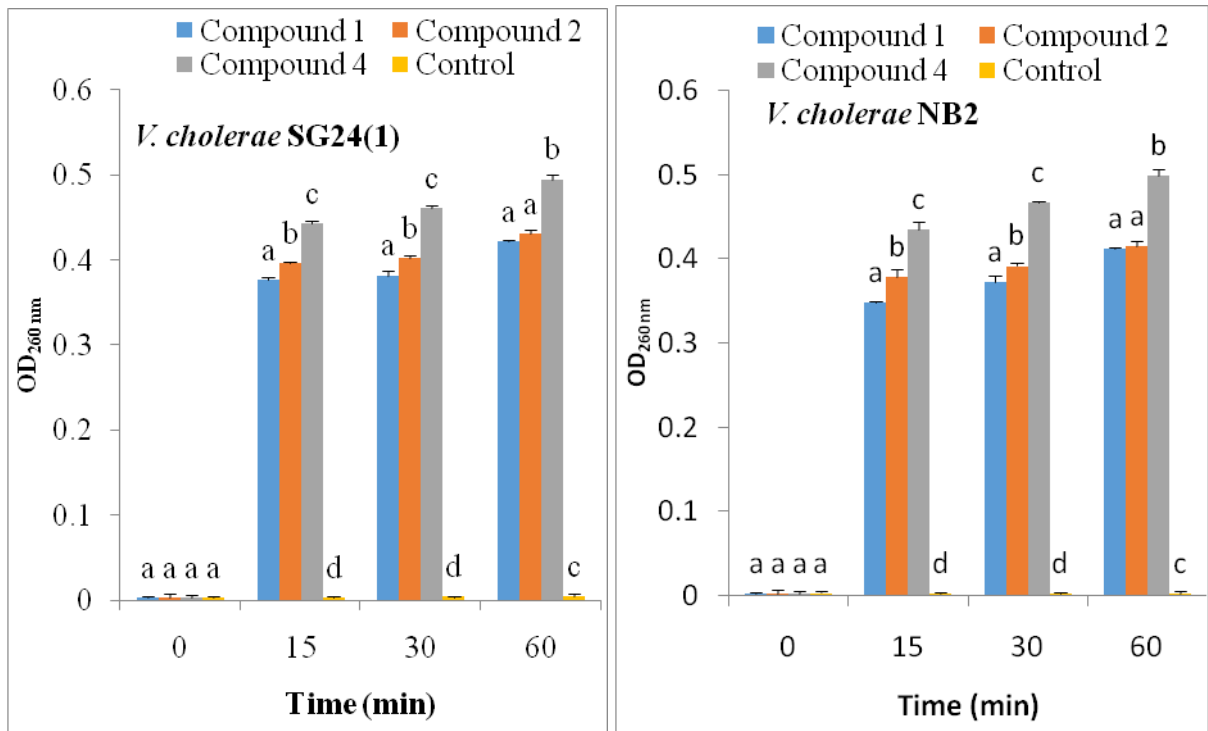
585 *G. grandulosum* Turill.

586 **1:**chrysoeriol-7-*O*- β -D-xyloside; **2:**luteolin-7-*O*- β -D-apiofuranosyl-(1 \rightarrow 2)- β -D-xylopyranoside; **3:** chrysoeriol-

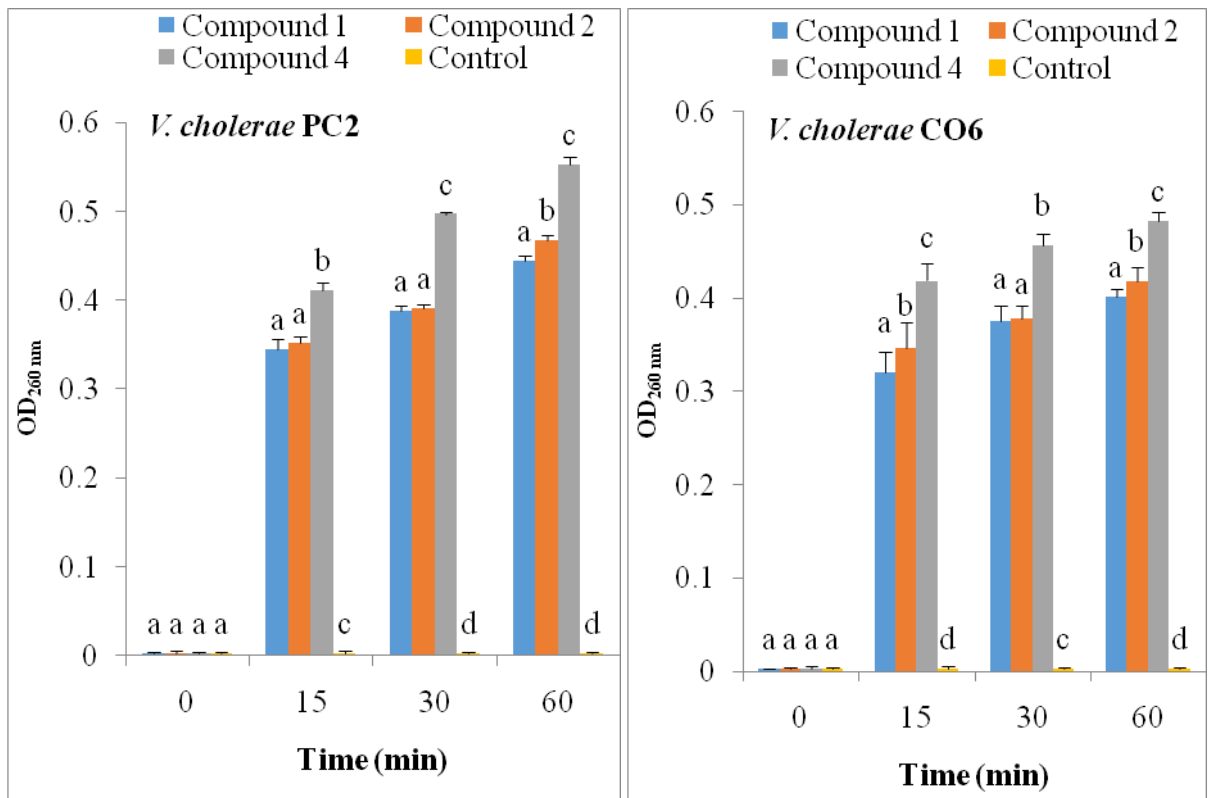
587 7-*O*- β -D-apiofuranosyl-(1 \rightarrow 2)- β -D-xylopyranoside; **4:** chrysoeriol-7-*O*- α -L-rhamnopyranosyl-(1 \rightarrow 6)- β -D-(4''-

588 hydrogensulfate) glucopyranoside; **5:**Isorhamnetin-3-*O*- α -L-rhamnopyranosyl-(1 \rightarrow 6)- β -D-glucopyranoside.

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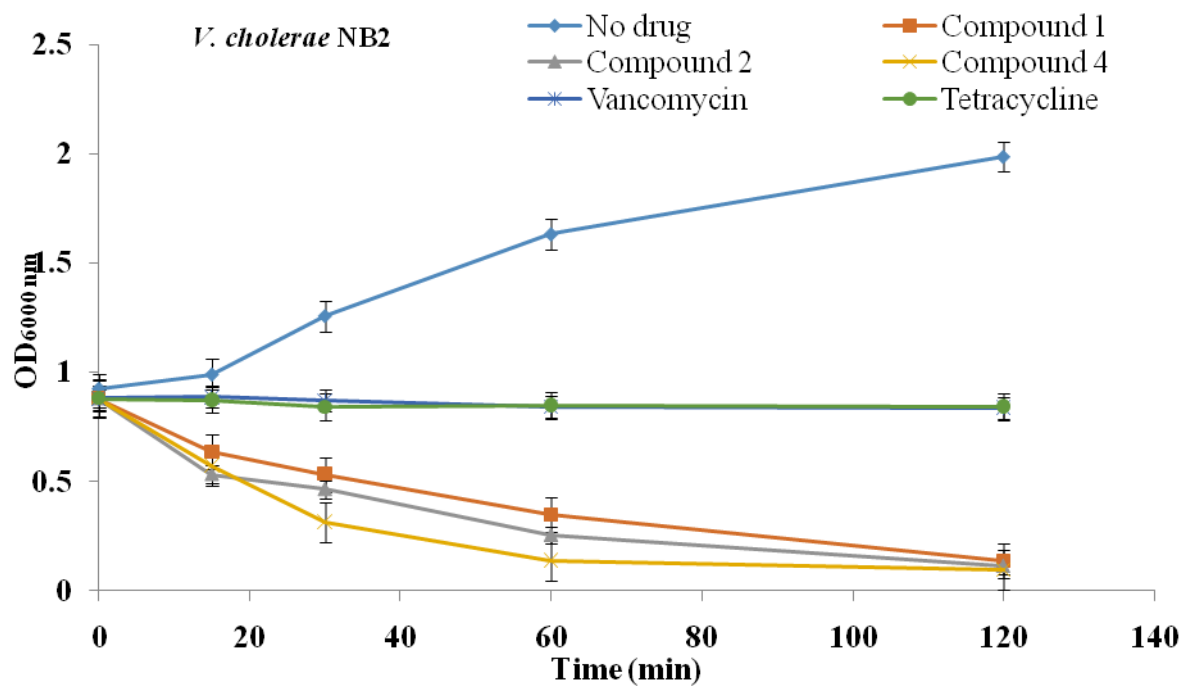


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591 **Fig. 2** Appearance of 260-nm-absorbing material in the filtrates of *V. cholerae* SG24 (1), PC2,
592 NB2 and CO6 after treatment with compounds 1, 2 and 4.

593 Bars represent the mean \pm standard deviation of the triplicate OD at each incubation time. At the same
594 incubation time, letters a-d indicate significant differences between samples according to one way ANOVA and
595 Waller Duncan test; $p < 0.05$.
596



597

598 **Fig. 3** Bacteriolytic effect of compounds **1**, **2** and **4** against *V. cholerae* NB2

599 Results represent the mean \pm standard deviation of the triplicate OD at each incubation time.

600

601 **Table 1.** Antimicrobial activities of extracts, isolated compounds and reference antimicrobial
 602 drugs.

Extracts/ Compounds	Inhibition parameters	V. <i>cholerae</i> SG24 (1)	V. <i>cholerae</i> CO6	V. <i>cholerae</i> NB2	V. <i>cholerae</i> PC2	<i>S. aureus</i> ATCC 25923	<i>C. albicans</i> ATCC 9002	<i>C.</i> <i>neoformans</i> IP95026
MeOH extract	MIC	512	512	256	512	256	>2048	>2048
	MMC	512	512	512	1024	512	/	/
	MMC/MIC	1	1	2	2	2	/	/
<i>n</i> -BuOH extract	MIC	256	256	128	128	128	2048	2048
	MMC	256	256	128	256	128	>2048	>2048
	MMC/MIC	1	1	1	2	1	/	/
EtOAc extract	MIC	64	128	64	64	64	1024	512
	MMC	128	128	64	64	64	1024	1024
	MMC/MIC	2	1	1	1	1	1	2
1	MIC	16	8	8	8	4	32	8
	MMC	16	8	16	8	8	64	8
	MMC/MIC	1	1	2	1	2	2	1
2	MIC	16	16	8	8	4	64	16
	MMC	32	16	16	8	8	64	32
	MMC/MIC	2	1	2	1	2	1	2
3	MIC	32	64	32	32	8	32	16
	MMC	64	128	32	64	16	32	16
	MMC/MIC	2	2	1	2	2	1	1
4	MIC	8	8	8	8	4	8	4
	MMC	16	8	8	8	4	8	8
	MMC/MIC	2	1	1	1	1	1	2
5	MIC	32	16	16	16	8	64	32
	MMC	32	16	16	16	8	128	64
	MMC/MIC	1	1	1	1	1	2	2
Ref*	MIC	32	4	16	16	0.5	0.5	0.25
	MBC	32	4	16	16	0.5	0.5	0.25
	MBC/MIC	1	1	1	1	1	1	1

603 /: not determined; MIC: Minimum Inhibitory Concentration; MMC Minimum Microbicidal Concentration; the

604 MIC and MMC were measured in µg/mL; *: amphotericin B for yeasts and ciprofloxacin for bacteria.

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606 **Table 2.** Antibacterial activities in terms of MIC ($\mu\text{g/mL}$) of compounds **1**, **3** and **4** under
 607 osmotic stress condition against bacterial strains.

Bacteria	Compound 1		Compound 2		Compound 4		Chloramphenicol		Vancomycin	
	0% NaCl	5% NaCl	0% NaCl	5% NaCl	0% NaCl	5% NaCl	0% NaCl	5% NaCl	0% NaCl	5% NaCl
<i>V. cholerae</i> SG24 (1)	16	8	16	16	8	4	4	1	16	64
<i>V. cholerae</i> CO6	8	4	16	4	8	2	16	2	16	32
<i>V. cholerae</i> NB2	8	4	8	2	8	2	64	1	32	64
<i>V. cholerae</i> PC2	8	4	8	2	8	2	16	1	32	64
<i>S. aureus</i>	4	2	4	2	4	1	32	0.5	0,5	1

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