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1 ***Carissa edulis* Vahl (Apocynaceae) extract, a medicinal plant of Benin pharmacopoeia,**
2 **induces potent endothelium-dependent relaxation of coronary artery rings**
3 **involving nitric oxide**

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26

27 **Abstract:**

28 Background: Hypertension is a major cardiovascular risk factor that affects most countries
29 including those of Africa. Although *Carissa edulis*, *Diodia scandens* and *Cleome gynandra* are
30 traditionally used in Benin as antihypertensive treatments with some efficacy mentioned by the
31 local population, their biological activity on the cardiovascular system remains poorly studied.

32 Aim: Therefore, this study investigated the vasoactivity of the plants and assessed the
33 underlying mechanisms using isolated arteries.

34 Study design: Aqueous-ethanolic extracts of aerial parts of *C. edulis*, *D. scandens* and *C.*
35 *gynandra* were prepared by maceration before being subjected to multi-step liquid-liquid
36 fractionation with solvents of increasing polarity. The vasoactivity of the extracts and fractions
37 were assessed on isolated porcine coronary artery and rat aorta using organ chambers, the role
38 of nitric oxide (NO) using N^G-nitro-L-arginine (NO synthase inhibitor), prostanoids using
39 indomethacin (cyclooxygenases inhibitor) and endothelium-dependent hyperpolarization using
40 TRAM-34 plus UCL 1684 (inhibitors of calcium-dependent K⁺ channels), and the vascular
41 uptake of polyphenols using Neu reagent.

42 Results: The aqueous-ethanolic crude extract of *C. edulis* (CECE) induced potent relaxations
43 that were exclusively endothelium-dependent and more pronounced than those to *D. scandens*
44 and *C. gynandra*. The *n*-butanolic fraction of *C. edulis* (CEBF) was more active than the
45 cyclohexane, dichloromethane, and ethyl acetate fractions. The relaxation induced by CECE
46 and CEBF were inhibited by N^G-nitro-L-arginine and affected neither by TRAM-34 plus UCL
47 1684 nor by indomethacin. CEBF induced sustained endothelium-dependent relaxations for at
48 least 60 min, and inhibited, in a concentration-dependent manner, contractions to KCl, CaCl₂,
49 U46619 and 5-HT in rings with endothelium. Analysis of CEBF by LC-HRMS indicated the
50 presence of polyphenols, terpenes, and alkaloids. Exposure of coronary artery and aorta rings
51 to CEBF caused the accumulation of polyphenols predominantly in the endothelium.

52 Conclusion: *C. edulis* leaf extract induced pronounced endothelium-dependent relaxations and
53 inhibited contractile responses by stimulating the endothelial formation of NO. LC-HRMS
54 analysis of the most active fraction, the butanolic fraction, revealed the presence of numerous
55 compounds including polyphenols, terpenes, and alkaloids. The polyphenols of CEBF
56 accumulated preferentially in the endothelium of the arterial wall. Thus, these observations
57 support the folkloric use of *C. edulis* in hypertension.

58 **Keywords:** Hypertension, *Carissa edulis*, Endothelium, Vasorelaxant activity, Nitric oxide

59
60

61 **List of abbreviations**

62 CaCl₂, Calcium dichloride; CECE, *C. edulis* crude extract; CECF, *C. edulis* cyclohexane
63 fraction; CEDF, *C. edulis* dichloromethane fraction; CEEF, *C. edulis* ethyl acetate fraction;
64 CEBF, *C. edulis* butanolic fraction; EDH, Endothelium-dependent hyperpolarization;
65 EPA:DHA, Eicosapentaenoic acid: docosahexaenoic acid; eNOS, Endothelial NO synthase;
66 IK_{Ca} channels, Intermediate-conductance calcium-activated potassium channels; KCl,
67 Potassium chloride; KH₂PO₄, Potassium dihydrogen phosphate; LC-HRMS, Liquid
68 chromatography-high resolution mass spectrometry; L-NA, N^G-nitro-L-arginine; L-NAME, L-
69 nitro-arginine methyl ester; MgSO₄, Magnesium sulfate; MeOH, Methanol; NaCl, Sodium
70 chloride; NaOH, Sodium hydroxide; NaHCO₃, Sodium bicarbonate; SK_{Ca} channels, Small
71 conductance calcium-activated potassium channels.

72

73 **Introduction**

74 Cardiovascular disease is the most prevalent disease worldwide (Mendis et al., 2007).
75 Although hypertension is a major cardiovascular risk factor identified initially in Western
76 industrialized countries (Maione et al., 2013), its' prevalence is increasing continuously in
77 developing countries and, in particular, in sub-Saharan Africa (Tokoudagba et al., 2010).
78 Studies conducted in 2008 in 12 departments of Benin on 6853 people aged 25-64 years old
79 showed that the prevalence of hypertension is 27.9% (Houinato et al., 2012). Additional studies
80 have been carried out in 2015 on 1777 people aged over 25 years in the population of Tanvè, a
81 rural area 150 km north of Cotonou, and on 717 people aged 18-64 years in the Atlantic region
82 of Benin. These studies showed a prevalence of hypertension of 32.9% in the Tanvè population
83 and 33.9% in the Atlantic region (Desormais et al., 2019; Houssou et al., 2020). It is estimated
84 that 60-90% of the general African population uses folk medicine in the management of
85 diseases especially when life-long medication is required such as in the case of hypertension
86 (Balogun and Ashafa, 2019), and also due to the easier availability and the lower cost compared
87 to conventional medicines (Houngbeme et al., 2014; Lawson et al., 2016; Nunes et al., 2015;
88 Sen and Chakraborty, 2017; Yuan et al., 2016). *Carissa edulis* (*C. edulis*), *Diodia scandens* (*D.*
89 *scandens*) and *Cleome gynandra* (*C. gynandra*) are three plants traditionally used in Benin for
90 the treatment of hypertension (U. Hounguè, personal communication). *C. edulis* is a perennial
91 thorny shrub of the family of Apocynaceae widespread in Africa, Australia, Vietnam, Yemen
92 and India (Al-Youssef and Hassan, 2014). The biological activities and isolated molecules of
93 *C. edulis* have been recently summarized in our review (Hounguè et al., 2022). *D. scandens*
94 (Rubiaceae) is an evergreen perennial herb, which has an alternate leaf arrangement, (Akpuaka

95 et al., 2018). *C. gynandra* is a plant of the family of Cappariaceae, which grows in most
96 tropical countries. The leaves and seeds of *C. gynandra* have long been used as indigenous
97 medicine for treatment of headaches and stomach aches (Narendhirakannan et al., 2007). The
98 three plants have been reported to have diuretic, analgesic, antioxidant, antimicrobial,
99 antidiabetic, and anticancer activities, and the characterization of their phytochemical
100 composition indicated the presence of flavonoids, phenolic compounds, chlorogenic acid
101 derivatives, lignans, sesquiterpenes, sterols, triterpenes, coumarins, and cardiac glycosides
102 (Chandradevan et al., 2020; Fanta Yadang et al., 2019; Bala et al., 2010; Ibrahim et al., 2010;
103 Tolo et al., 2006; El-Fiky et al., 1996; Akah et al., 1993; Akpuaka et al., 2018; H Jorum and M
104 Piero, 2016; Sango et al., 2016; Ya'u et al., 2013). Acute oral toxicity studies of the leaves and
105 roots of the three plants revealed no sign of toxicity, mortality or reduction of body weight in
106 adult Wistar rats up to 2000 mg/kg for *C. edulis* and *C. gynandra*, and 800 mg/kg for *D.*
107 *scandens* (Dossou-Yovo et al., 2021; Asiedu-Larbi et al., 2020; Osseni et al., 2017, 2016; Shaik
108 et al., 2013). The possibility that the three plants affect vascular tone that contributes to regulate
109 vascular resistance and, hence, blood pressure, remains poorly studied. Such a hypothesis is
110 supported by the fact that numerous natural products including grape-derived polyphenols and
111 *Crataegus spp.* (Hawthorn) have been shown to cause endothelium-dependent relaxations of
112 isolated arteries by stimulating the endothelial formation of nitric oxide (NO), a potent inhibitor
113 of vascular tone and platelet activation (Schini-Kerth et al., 2010a; Vanhoutte, 2018).

114 Thus, the aim of the study is to investigate the potential of extracts prepared from leaves
115 of the three selected plants to affect vascular tone and, if so, to clarify the role of the
116 endothelium and the underlying mechanisms.

117

118 **Materials and methods**

119 **Plant material and extraction**

120 The leaves of *C. edulis*, *D. scandens* and *C. gynandra* were collected at Togbin,
121 Godomey, Benin in September 2017 and identified by Pr Hounnankpon Yedomonhan,
122 Professor of Botany at the University of Abomey-Calavi, Benin. The reference specimens were
123 deposited at the National Herbarium of the University of Abomey-Calavi, Benin. The samples
124 were dried, powdered (100 g), and macerated at room temperature with ethanol/water (6:4, v/v)
125 (3x300 ml for 72 h each). For each plant, the filtered extraction solutions were combined and
126 evaporated at reduced pressure to produce a dry extract with a yield of about 25%, 21% and
127 23% for *C. edulis*, *D. scandens* and *C. gynandra*, respectively. The aqueous ethanolic extract

128 of the plant showing the greatest vasorelaxation was successively fractionated using cyclohexane,
129 dichloromethane, ethyl acetate and then butanol.

130 The *C. edulis* crude extract (CECE, 10 g) was completely dissolved in water (100 ml) followed
131 by the addition of the same volume of cyclohexane before a 24-h incubation period. This
132 sequence was repeated three times. Following evaporation, the cyclohexane fraction of *C.*
133 *edulis* (CECF) was collected and amounted to about 66 mg. The remaining aqueous layer was
134 extracted first with dichloromethane, then with ethyl acetate and lastly with butanol (100 ml
135 each). Each extraction step involved a 24-h incubation period that was repeated three times.
136 Following evaporation, the dichloromethane fraction of *C. edulis* (CEDF) provided about 16
137 mg, the ethyl acetate fraction (CEEF) about 0.43 g, and the butanolic fraction (CEBF) about
138 1.04 g. All extracts were kept at 4 °C before use. The CECE and the different fractions were
139 fully solubilized in a mixture of ethanol water 60:40 v/v to prepare a 10 mg/ml stock solution.
140 Thereafter, the 10 mg/ml solution was diluted in water to obtain the appropriate solutions.

141 **Chemicals and drugs**

142 All pharmacological reagents were from Sigma-Aldrich (Sigma-Aldrich Chemie SARL,
143 St Quentin Fallavier, France). De-ionised water was filtered through a Direct-Q UV (Millipore)
144 station, isopropanol and methanol were purchased from Fisher Chemicals (Optima ® LC/MS
145 grade). NaOH was obtained from Agilent Technologies, and acetic acid, formic acid,
146 cyclohexane, dichloromethane, ethyl acetate and butanol from Sigma-Aldrich. All chemicals
147 were of analytical grade.

148 **Liquid chromatography coupled to high resolution mass spectrometry analysis**

149 CEBF was analyzed using liquid chromatography coupled to high resolution mass
150 spectrometry (LC-HRMS) on an UltiMate 3000 system (Thermo) coupled to an Impact II
151 (Bruker) quadrupole time-of-flight (Q-TOF) spectrometer. Chromatographic separation was
152 performed on an Acquity UPLC ® BEH C18 column (2.1 x 100 mm, 1.7 µm, Waters) equipped
153 with an Acquity UPLC ® BEH C18 pre-column (2.1 x 5 mm, 1.7 µm, Waters) using a gradient
154 of solvents A (Water, 0.1% formic acid) and B (MeOH, 0.1% formic acid). Chromatography
155 was carried out at 35 °C with a flux of 0.3 ml.min⁻¹, starting with 5% B for 2 min, reaching
156 100% B at 10 min, holding 100% for 3 min and coming back to the initial condition of 5% B
157 in 2 min, for a total run time of 15 min. Extract was kept at 4 °C, 5 µl were injected in full loop
158 mode with a washing step after sample injection with 150 µl of wash solution (H₂O/MeOH,
159 90/10, v/v). The spectrometer was equipped with an electrospray ionization (ESI) source and
160 operated in positive ion mode on a mass range from 20 to 1000 Da with a spectra rate of 2 Hz
161 in AutoMS/MS fragmentation mode. The end plate offset was set at 500 V, capillary voltage at

162 2500 V, nebulizer at 2 Bar, dry gas at 8 l.min⁻¹ and dry temperature at 200 °C. The transfer time
163 was set at 20-70 μs and MS/MS collision energy at 80-120% with a timing of 50-50% for both
164 parameters. The MS/MS cycle time was set to 3 sec, absolute threshold to 816 cts and active
165 exclusion was used with an exclusion threshold at 3 spectra, release after 1 min and precursor
166 ion was reconsidered if the ratio current intensity/previous intensity was higher than 5. A
167 calibration segment was included at the beginning of the runs allowing the injection of a
168 calibration solution from 0.05 to 0.25 min. The calibration solution used was a fresh mix of 50
169 ml isopropanol/water (50/50, v/v), 500 μl NaOH 1 M, 75 μl acetic acid and 25 μl formic acid.
170 The spectrometer was calibrated on the [M+H]⁺ form of reference ions (57 masses from *m/z*
171 22.9892 to *m/z* 990.9196) in high precision calibration (HPC) mode with a standard deviation
172 below 1 ppm before the injections for each polarity mode, and re-calibration of each raw data
173 was performed after injection using the calibration segment.

174 **Metabolite identification**

175 Raw data were processed in MetaboScape 4.0 software (Bruker): molecular features
176 were considered and grouped into buckets containing one or several adducts and isotopes from
177 the detected ions with their retention time and MS/MS information when available. The
178 parameters used for bucketing are a minimum intensity threshold of 10 000, a minimum peak
179 length of 3 spectra, a signal-to-noise ratio (S/N) of 3 and a correlation coefficient threshold set
180 at 0.8. The [M+H]⁺, [M+Na]⁺ and [M+K]⁺ ions were authorized as possible ions. Replicate
181 samples were grouped and only the buckets found in 80% of the samples of one group were
182 extracted from the raw data. The obtained list of buckets was annotated using SmartFormula to
183 generate raw formula based on the exact mass of the primary ions and the isotopic pattern. The
184 maximum allowed variation on the mass ($\Delta m/z$) was set to 3 ppm, and the maximum mSigma
185 value (assessing the good fitting of isotopic patterns) was set to 30. To put a name on the
186 obtained formulae, an analyte list was derived from KNApSAcK database
187 (<http://www.knapsackfamily.com/KNApSAcK/>). The parameters used for the annotation with
188 the analyte lists are the same as for SmartFormula annotation.

189 **Determination of total phenols content**

190 The total phenols content of CECE and its fractions were determined in triplicate and
191 expressed as gallic acid equivalents (GAE) using the Folin–Ciocalteu reagent (Singleton and
192 Rossi, 1965) that was adapted for microplate reader (Auger et al., 2010). The Folin-Ciocalteu
193 reagent contains a mixture of phosphotungstic and phosphomolybdic acids, which will react
194 with phenols to form a blue complex visible at 730 nm. In each well of a 96 wells plate, 30 μl
195 of the dilute extract or fraction were deposited, to which 10 μl of Folin-Ciocalteu reagent and

196 30 μ l of Na_2CO_3 (20 %) were added. Then, the total volume was adjusted to 200 μ l with distilled
197 water. Gallic acid was used as the standard. After 30 min at room temperature, the absorbance
198 was measured at 730 nm using an IEMS Lab System reader, MF spectrophotometer. The
199 calibration curve was constructed using a standard solution of gallic acid (0 to 100 mg/ml).
200 Blank solution: water-ethanol instead of test solution was used. Each assay was carried out in
201 triplicate and expressed as mg/ml gallic acid equivalents.

202 **Vascular reactivity studies**

203 Vascular reactivity studies were performed using left circumflex porcine coronary
204 arteries and the thoracic aorta of 20-week-old rats. Porcine hearts were collected from the local
205 slaughterhouse (Socopa, Holtzheim). Male Wistar rats (Janvier-labs, Le Genest-Saint-Isle,
206 France) were kept in the animal facility with controlled temperature (22 °C), 12 h light/dark
207 cycle and were given free access to standard food (SAFE[®] A04) and water until the age of 20
208 weeks. Both arteries were cleaned of connective tissue and cut into rings (2-3 mm in length).
209 Rings were suspended in organ baths containing oxygenated (95% O_2 , 5% CO_2) Krebs
210 bicarbonate solution (composition in mM: NaCl 119, KCl 4.7, KH_2PO_4 1.18, MgSO_4 1.18,
211 CaCl_2 1.25, NaHCO_3 25 and D-glucose 11, pH 7.4, 37 °C) for the assessment of changes in
212 isometric tension. The rings were stretched to an optimal resting tension of 5 g for the porcine
213 coronary artery and 2 g for the thoracic aorta. After the equilibration period, the rings were
214 exposed to a high K^+ -containing Krebs bicarbonate solution (80 mM) until reproducible
215 contractile responses were obtained. To assess the endothelial function of the porcine coronary
216 arteries, the rings were precontracted sub-maximally with U46619 (a thromboxane A_2 receptor
217 agonist) before the induction of a relaxation to bradykinin (0.3 μ M). To assess the vasorelaxant
218 properties of the extracts, rings were contracted (about 80% of the maximal contraction) with
219 U46619 before construction of a concentration-relaxation curve to an extract. For assessment
220 of the inhibition of contractile responses, rings with endothelium were exposed to CEBF (1, 3
221 and 10 μ g/ml) for 30 min before construction of a concentration-contraction curve to either
222 KCl, U46619, CaCl_2 in the presence of 40 mM KCl or serotonin. To characterize the different
223 pathways mediating relaxation, rings were incubated for 30 min with a pharmacological agent
224 before the construction of concentration-relaxation curve to an extract. The role of NO-
225 mediated relaxation was studied in rings incubated with N^G -nitro-L-arginine (L-NA, 300 μ M,
226 NOS inhibitor). To study the role of prostanoids, rings were incubated with indomethacin (10
227 μ M, cyclooxygenases inhibitor). To study endothelium-dependent hyperpolarization (EDH)-
228 mediated relaxation, rings were incubated with the combination of TRAM-34 plus UCL 1684
229 (1 μ M each, inhibitors of IK_{Ca} and SK_{Ca} , respectively). To assess the endothelial function of the

230 rat thoracic aorta, rings were precontracted sub-maximally with phenylephrine (PE, 1 μ M)
231 before the induction of a relaxation to acetylcholine (ACh, 1 μ M). Rings were then contracted
232 to about 80 % of the maximal contraction with PE before construction of a concentration-
233 relaxation curve to either ACh or CEBF.

234 ***Ex vivo* polyphenols uptake experiment**

235 Segments of porcine coronary artery and rat thoracic aorta were cleaned of connective
236 tissue and cut into rings (2-3 mm in length). Then, they were washed in Krebs solution and
237 placed in a 24 wells plate containing MCDB131 culture medium (Gibco, ThermoFisher
238 Scientific, Illkirch, France) supplemented with fungizone (250 μ g/ml), penicillin (100 UI/ml),
239 streptomycin (100 UI/ml), L-glutamine (2 mM, all from Lonza Levallois-Perret, France).
240 Coronary artery and aorta rings were incubated with CEBF (30, 60, 80 or 100 μ g/ml) for 3 min
241 at 37 °C with 5% CO₂. They were then washed twice with PBS before further incubation in
242 PBS containing 0.4 % of 2-aminoethyl diphenyl borate for 5 min at 37 °C to determine the
243 uptake of polyphenols according to Jin et al., 2013 with some modifications. Subsequently,
244 rings were washed with cold PBS, snap-frozen in histomolds containing frozen section media
245 (FSC 22, Leica Biosystems, Nanterre, France) in liquid nitrogen. Thereafter, they were
246 cryosectioned at 14 μ m and mounted on Superfrost plus slides (ThermoFisher) with Dako
247 medium (fluorescence editing medium, Dako, Agilent Technologies France, Les Ulis, France)
248 and dried for 20 min at room temperature. Slides were then analyzed the same day using a
249 confocal laser scanning microscope (Leica TSC SPE). Quantitative analysis of fluorescence
250 was performed using Image J software (version 1.6 for Windows, NIH).

251 **Statistical analysis**

252 Data are expressed as mean \pm standard error mean (S.E.M.) of n different experiments
253 using GraphPad Prism 8 (GraphPad Software, Inc., San Diego, CA, USA). Normality of data
254 was assessed using Shapiro-Wilks, Anderson-Darling, and Kolmogorov-Smirnov tests.
255 Vascular reactivity results were analyzed using a mixed-effect model for repeated measures and
256 quantitative confocal microscopy results using one-way Anova test. The Bonferroni *post-*
257 *hoc* test (comparison of selected pairs) was used to identify significance between groups. The
258 difference between groups was considered significant for a $P < 0.05$.

259

260 **Results**

261 **Total phenols content**

262 The total phenols content was 856.66 ± 23.33 mg of gallic acid equivalent (GAE) *per*
263 gram for CECE; 106.66 ± 18.55 mg GAE/g for CECE; 196.66 ± 14.53 mg GAE/g for CEDF;
264 580.00 ± 5.77 mg GAE/g for CEEF and 793.33 ± 17.63 mg GAE/g for CEBF.

265

266 **Identification of CEBF-containing metabolites**

267 Analysis of the fraction showing the greatest vasorelaxant activity, CEBF, by LC-HRMS
268 revealed the presence of numerous compounds including polyphenols, terpenes, and alkaloids
269 (Table 1). Table 1 provides the list of compounds including the name, the molecular formula,
270 the measured *m/z* (masse/charge) ratio and the mSigma. The identification of the name of each
271 compound corresponded to the level 4, and the molecular formula to the level 3 (Schymanski
272 et al., 2014).

273 **Vasorelaxant effect on porcine coronary artery rings**

274 The aqueous ethanolic crude extract of *C. edulis*, *D. scandens* and *C. gynandra* caused
275 greater concentration-dependent relaxations in endothelium intact compared to endothelium
276 denuded coronary artery rings (Figures 1A-C). In addition, the CECE induced greater
277 endothelium-dependent relaxations than that of *D. scandens* and *C. gynandra* (Figures 1A-C).
278 Maximal relaxation was observed in response to 100 μ g/ml of CECE (Figure 1A). All
279 subsequent experiments were performed with *C. edulis*, the most active plant, to characterize
280 the underlying mechanism.

281 CECE was successively fractionated with solvents of increasing polarity including
282 cyclohexane, dichloromethane, ethyl acetate and butanol, and their vasorelaxant activity was
283 assessed. The *n*-butanolic fraction showed a greater relaxing activity than the crude extract
284 while the ethyl acetate, dichloromethane and cyclohexane extracts were significantly less active
285 (Figure 2A).

286 Compared to bradykinin, CEBF induced endothelium-dependent relaxations that
287 persisted at least over 60 min whereas those to bradykinin returned gradually to the pre-
288 contractile level after 5 min (Figure 2B).

289 Relaxation to CECE and CEBF were minimally affected by the presence of
290 indomethacin (inhibitor of cyclooxygenases), and by TRAM-34 plus UCL 1684 (inhibitors of
291 EDH-mediated relaxation, Figures 2C & D). In contrast, N^{G} -nitro-L-arginine, a competitive NO
292 synthase inhibitor markedly reduced relaxations to the crude extract and the butanolic fraction
293 indicating the predominant involvement of NO (Figures 2C & D).

294 **CEBF inhibits KCl, CaCl₂, U46619 and serotonin-induced contractile responses: Role of**
295 **the endothelial formation of NO**

296 CEBF significantly inhibited the contractile responses of coronary artery rings with
297 endothelium to different vasocontractile agonists including KCl, CaCl₂ in the presence of 40
298 mM of KCl, U46619 and serotonin in a concentration-dependent manner (Figure 3). At a
299 concentration of 10 µg/ml, CEBF significantly shifted to the right and reduced maximal
300 contractile responses to KCl and CaCl₂ whereas those to U46619 and serotonin were markedly
301 reduced (Figure 3). In the presence of N^G-nitro-L-arginine, contractile responses to serotonin
302 were significantly increased, and the inhibitory effect of CEBF at 10 µg/ml was not observed
303 indicating that NO synthase-derived NO blunted contractile responses.

304 **Vasorelaxant effect on rat aortic rings**

305 Like coronary rings, CEBF induced pronounced relaxations of precontracted aortic rings
306 with endothelium compared to those without endothelium in rats (Figure 4).

307 **Preferential accumulation of *C. edulis* polyphenols in the endothelium of porcine coronary**
308 **artery and rat aorta**

309 Exposure of both porcine coronary artery and rat aortic rings to CEBF indicated a
310 concentration-dependent accumulation of polyphenols as assessed using Neu reagent that was
311 more pronounced in the endothelium than the vascular smooth muscle (Figures 5A & B).
312 Moreover, the intensity of the endothelial accumulation of polyphenols was greater in the rat
313 thoracic aorta (636.4 ± 61.11 AU) than in porcine coronary artery rings (348.9 ± 40.5 AU) at
314 100 µg/ml (Figure 5).

315

316 **Discussion**

317 The present findings indicate that the aqueous ethanolic extract of *C. edulis* leaves
318 induced greater relaxations of coronary artery rings with endothelium than those of *D. scandens*
319 and *C. gynandra*. In contrast to rings with endothelium, all three extracts caused only small
320 relaxations in rings without endothelium indicating that the vasorelaxant effect is
321 predominantly mediated by the endothelium. Furthermore, the *n*-butanolic fraction of *C. edulis*
322 was more potent to induce endothelium-dependent relaxations than the crude extract and the
323 cyclohexane, dichloromethane and ethyl acetate fractions. CECE and CEBF-induced
324 relaxations in coronary artery rings with endothelium were markedly reduced by L-NA, an
325 inhibitor of NO synthase, and minimally affected by inhibition of either EDH-mediated
326 relaxation using the combination of TRAM-34 plus UCL 1684 or cyclooxygenases using

327 indomethacin. Thus, the vasorelaxant activity of CECE and CEBF involves predominantly the
328 endothelial formation of NO.

329 Endothelium-dependent relaxations are observed in response to a variety of stimulators
330 including mechanical forces and humoral factors (Vanhoutte, 2018). In most types of
331 conductance arteries, endothelium-dependent relaxations involve mostly NO whereas in
332 resistance arteries and in large coronary arteries NO and EDH are often equally important
333 (Vanhoutte, 2018). Endothelium-derived NO plays a pivotal role in the regulation of vascular
334 homeostasis by regulating vascular tone, and also by inhibiting platelet adhesion and
335 aggregation, and the adhesion of monocytes triggered by a variety of proatherogenic factors
336 (Boo and Jo, 2003; Vanhoutte, 2018). Besides physiological activators, endothelium-dependent
337 relaxations have also been observed in response to natural products such as a polyphenol-rich
338 red wine extract (Ndiaye et al., 2005), an extract of *Dicksonia sellowiana* (Rattmann et al.,
339 2012) and as observed in the present study the polyphenol-rich CECE in porcine coronary
340 arteries and rat aorta. Characterization of the endothelium-dependent relaxations in coronary
341 arteries indicated the involvement of NO and also, to some extent, EDH in response to the red
342 wine extract (Ndiaye et al., 2005) and the *Dicksonia sellowiana* extract (Rattmann et al., 2012).
343 In contrast, the endothelium-dependent relaxation to CECE was exclusively mediated by NO.
344 Of interest, the *C. edulis*-induced endothelium-dependent relaxation is a sustained event in
345 contrast to that induced by the physiological agonist bradykinin, and, hence, might be more
346 appropriate for a persistent control of vascular tone. Natural products have been shown to cause
347 endothelium-dependent relaxations in a variety of blood vessels and species including rat aortic
348 rings (Diebolt et al., 2001), porcine coronary artery rings (Ndiaye et al., 2005), and human
349 internal mammary artery rings (Zgheel et al., 2019). Besides blood vessels, natural products
350 might also be of interest to regulate the tone of the respiratory cartilaginous tube since a hexane
351 extract of *Achillea millefolium* caused relaxations of rat tracheal rings by a non-competitive
352 antagonism of muscarinic receptors, calcium channel blockade, and release of NO (Arias-Durán
353 et al., 2020). In addition to regulation of vascular tone, *C. edulis* has been shown to have
354 antioxidant and anti-inflammatory properties (Al-Youssef and Hassan, 2012; Fanta Yadang et
355 al., 2019). Liu et al. studied the effect of *C. edulis* root bark on cyclooxygenase-2 (COX-2)
356 activity and observed that the crude extract and four fractions inhibited COX-2 to varying
357 degrees (Liu et al., 2021). Polyphenols have been shown to stimulate endothelial NO synthase
358 to promote the formation of NO, and to up-regulate the expression of endothelial NO synthase
359 in endothelial cells (Schini-Kerth et al., 2010b). Different pathways have been involved in the
360 endothelial formation of NO in response to polyphenols including the redox-sensitive activation

361 of Src/phosphatidylinositol 3-kinase/Akt pathway, the calcium/calmodulin pathway, the
362 activation of estrogen receptors, the 5' adenosine monophosphate-activated protein kinase and
363 sirtuin 1/Krüppel-like Factor 2 pathways (Anter et al., 2004; Oak et al., 2018).

364 Contractile responses of blood vessels to KCl are mediated predominantly by membrane
365 depolarization and the subsequent influx of calcium through voltage-operated calcium channels
366 (Kuriyama et al., 1998). In case of G-protein coupled membrane receptor-mediated contractile
367 responses, the activator calcium signal involves both extracellular Ca²⁺ influx through receptor-
368 operated calcium channels and voltage-dependent calcium channels, and also the release of
369 Ca²⁺ from intracellular Ca²⁺ stores (Broekaert and Godfraind, 1979). CEBF inhibited in a
370 concentration-dependent manner contractile responses in rings with endothelium induced either
371 by KCl, CaCl₂, U46619 or serotonin indicating that the inhibitory effect of CEBF is effective
372 against several signal transduction pathways leading to the Ca²⁺-dependent activation of
373 contractile proteins. A similar effect has also been observed previously in coronary artery rings
374 in response to the butanolic fraction of a *Berberis orthobotrys* root extract (Alamgeer et al.,
375 2016). CEBF inhibited very effectively contractile responses to U46619 and serotonin. The
376 inhibitory effect of CEBF on contractile responses to serotonin required the presence of a
377 functional endothelium and was prevented by an inhibitor of NO synthase, implying a major
378 role of the endothelial formation of NO. A previous study has also shown that the omega-3
379 polyunsaturated fatty acid formulation EPA:DHA 6:1 strongly inhibited serotonin-induced
380 contractions in porcine coronary artery rings mostly through an increased endothelial formation
381 of NO (Zgheel et al., 2019).

382 Analysis of the CECE and CEBF has indicated that they are rich in polyphenols.
383 Previous studies have reported that polyphenols are the major active ingredients in a wide range
384 of medicinal plants used for vascular protection, and involved in endothelium-dependent
385 relaxations of isolated arteries (Andriambelason et al., 1997; Diebolt et al., 2001; Ndiaye et al.,
386 2005; Tokoudagba et al., 2010). Plant-derived polyphenols can induce endothelium-dependent
387 relaxations following the redox-sensitive activation of Src kinase resulting in the activation of
388 the PI3-kinase/Akt pathway, and the subsequent phosphorylation of eNOS at the activator site
389 Ser1177 (Anselm et al., 2009). Studies evaluating sixty plants with validated
390 antihypertensive/vasorelaxant activity including *Achillea millefolium* and *Ailanthus excelsa*
391 indicated that the biological activity is mostly due to polyphenols followed by terpenes and
392 alkaloids (Maione et al., 2013). Moreover, the antihypertensive/vasorelaxant effect has been
393 reported to include several mechanisms including inhibition of angiotensin-converting enzyme
394 and calcium channels, and activation of endothelial NO synthase and adenylyl cyclase (Maione

395 et al., 2013). Polyphenols of CEBF include flavonoids, phenolic acids, coumarins and
396 anthraquinones. A previous study indicated that flavonoids present in the ethyl acetate extract
397 of *C. edulis* are mainly in the form of glycosylated derivatives (Al-Youssef and Hassan, 2017).
398 Flavonoids have been reported previously to have vasoactive properties. Indeed, luteolin and
399 quercetin from the buds of *Coreopsis tinctoria* caused relaxation of rat aortic rings (Sun et al.,
400 2013), and flavonoids of *Elsholtzia splendens* relaxed aortic rings in both the presence and
401 absence of the endothelium (Wang et al., 2014). Paredes et al. evaluated the antihypertensive
402 effect of several flavonoid extracts (lemon, grapefruit + bitter orange, and cocoa) using a
403 hypertensive rat model after chronic (6 weeks) infusion of the NO synthase inhibitor, L-NAME.
404 The results showed that flavonoid-rich extracts decreased blood pressure induced by chronic
405 administration of L-NAME in rats (Paredes et al., 2018).

406 Although several polyphenol-rich products have been shown to stimulate the endothelial
407 formation of NO, little is known regarding their interaction with endothelial cells. Jin et al.
408 showed that the delphinidin-3-*O*-glucoside entered into endothelial cells *via* a transport
409 mechanism involving the sodium-glucose co-transporter 1 in a temperature and time-dependent
410 manner (Jin et al., 2013). Therefore, the accumulation of *C. edulis* polyphenols in the arterial
411 wall was assessed using the Neu reagent. The findings indicated that *C. edulis* polyphenols
412 accumulate in the porcine coronary artery and the rat aortic wall, and that this effect occurs
413 preferentially in the endothelium.

414 Analysis of CEBF by LC-HRMS revealed the presence, besides polyphenols, also of
415 vasoactive terpenes, and alkaloids. Cardoso Lima et al. showed that both oxygenated terpenes
416 and non-oxygenated terpenes promoted endothelium-dependent relaxations of rat mesenteric
417 artery rings (Cardoso Lima et al., 2012). The alkaloids quindolinone and cryptolepine salt, two
418 indoquinoline alkaloids isolated and identified from aerial parts of *Sida rhombifolia*, showed
419 endothelium-dependent relaxations in rodent mesenteric artery rings (Chaves et al., 2017).
420 Wicha et al. studied the effects of neferin, an isoquinoline, on aortic rings from hypertensive
421 rats after chronic (4 weeks) administration of L-NAME. They showed that this molecule
422 induced relaxations mediated by the endothelial NO synthase/NO/soluble guanylyl pathway
423 (Wicha et al., 2020).

424 In conclusion, the present findings indicate that an extract of *C. edulis* leaves is a potent
425 vasorelaxant natural product and inhibitor of contractile responses mostly by stimulating the
426 endothelial formation of NO. The vasorelaxant activity is mainly observed with the *n*-butanolic
427 extract of *C. edulis* and is a persistent effect promoting a sustained control of vascular tone.
428 CEBF is rich in polyphenols, which are active natural compounds of a wide range of medicinal

429 plants used for vascular protection, and also contains terpenes and alkaloids that have been
430 shown to have some vasorelaxant properties. The vasorelaxant effect of *C. edulis* is strictly
431 endothelium dependent and is associated with the accumulation of polyphenols preferentially
432 in the endothelium. Thus, the vasorelaxant activity of *C. edulis* can contribute to explain its
433 traditional use for the treatment of hypertension.

434

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448

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450 We wish to confirm that there are no known conflicts of interest associated with this publication
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453

454 **Reference**

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675

676 **Figure legends**

677 **Figure 1.** The crude extract of *C. edulis* (A) induced greater endothelium-dependent relaxations
678 than that of *Diodia scandens* (B) and *Cleome gynandra* (C) in porcine coronary artery rings.
679 Results are shown as means \pm S.E.M. of 4-6 experiments. * $P < 0.05$ vs respective control with
680 endothelium.

681
682 **Figure 2.** (A) The butanolic fraction of *C. edulis* (CEBF) is more active than the crude extract
683 and the other types of fractions. (B) CEBF induced endothelium-dependent relaxations that
684 lasted for at least 60 min whereas those to bradykinin were transient and returned to the pre-
685 contractile level after 5 min. Both the crude extract- (C) and the butanolic fraction- (D) induced
686 relaxations involved predominantly NO in porcine coronary arteries with endothelium. Results
687 are shown as means \pm S.E.M. of 4-5 experiments. * $P < 0.05$ vs the respective control.

688
689 **Figure 3.** The butanolic fraction of *C. edulis* inhibits contractile responses to KCl (A), CaCl₂
690 in the presence of 40 mM of KCl (B), U46619 (C), and serotonin (D) in porcine coronary artery
691 rings with endothelium. Role of endothelium-derived NO was assessed using N^G-Nitro-L-
692 arginine (300 μ M) (E). Results are shown as means \pm S.E.M. of 4-6 experiments. * $P < 0.05$ as
693 compared to control.

694
695 **Figure 4.** The butanolic extract of *C. edulis* induced relaxations of rat aortic rings with but not
696 without endothelium. Results are shown as means \pm S.E.M. of 4-5 experiments. * $P < 0.05$ vs
697 control with endothelium.

698
699 **Figure 5.** Exposure of (A) porcine coronary artery rings with endothelium and (B) rat aortic
700 rings with endothelium to the butanolic fraction of *C. edulis* caused the accumulation of
701 polyphenols predominantly in the endothelium in a concentration-dependent manner. Rings
702 were exposed to the butanolic fraction for 3 min before being exposed to the Neu reagent for 5
703 min to assess the uptake of polyphenols using confocal microscopy. Results are shown as means
704 \pm S.E.M. of 4 experiments. * $P < 0.05$ vs control.

Table 1: Analysis of CEBF chemical compounds by LC-HRMS.

Name	Molecular formula	Exact Mass (g/mol)	Measured <i>m/z</i>	Ion	$\Delta m/z$ (ppm)	mSigma
Ethyl beta-D-glucopyranoside	C ₈ H ₁₆ O ₆	208.094688	247.05802	[M+K] ⁺	0.346	3.8
Germanaism D	C ₂₇ H ₃₀ O ₁₆	610.15338491	611.16146	[M+H] ⁺	1.456	9.0
Genistein 7-O-(2''-p-coumaroylglucoside)	C ₃₀ H ₂₆ O ₁₂	578.142426296	579.15025	[M+H] ⁺	0.894	5.0
alpha-Ketoglutaric acid	C ₅ H ₆ O ₅	146.021523	129.01819	[M-H ₂ O+H] ⁺	-0.344	6.0
Cinchonanin IIa	C ₃₉ H ₃₂ O ₁₅	740.17412	741.18305	[M+H] ⁺	2.184	8.0
Randioside	C ₁₆ H ₂₀ O ₁₀	372.10564683	373.11326	[M+H] ⁺	0.825	16.5
trans-2-Hexenyl acetate	C ₈ H ₁₄ O ₂	142.09938	143.10667	[M+H] ⁺	0.184	4.3
Plumiepoixide	C ₂₁ H ₂₆ O ₁₃	486.13734092	487.17514	[M+H] ⁺	1.246	10.5
5,7,4'-Trihydroxyisoflavone 7-O-beta-D-glucopyranoside-4'-O-[(beta-D-gucopyranosyl)-(1->2)-beta-D-glucopyranoside]	C ₃₃ H ₄₀ O ₂₀	756.21129372	757.22015	[M+H] ⁺	2.088	9.0
Tectorigenin 7-O-gentiobioside	C ₂₈ H ₃₂ O ₁₆	624.169035	625.17731	[M+H] ⁺	1.300	5.6
Patuletin 7-[6''-(2-methylbutyryl)-glucoside]	C ₂₇ H ₃₀ O ₁₄	578.523	579.17190	[M+H] ⁺	1.684	18.2
Catechin 3-O-rutinoside	C ₂₇ H ₃₄ O ₁₅	598.18977	599.19802	[M+H] ⁺	1.746	19.7
6-C-Glucosylquercetin	C ₂₁ H ₂₀	464.095490	465.10325	[M+H] ⁺	0.852	11.0
Cimicifugic acid E	C ₂₁ H ₂₀ O ₁₀	432.105647				
Foliasalacioside B2	C ₂₄ H ₄₀ O ₁₁	504.25706212	505.26442	[M+H] ⁺	0.041	7.6
7-Hydroxy-10-deoxyeucommiol	C ₉ H ₁₆ O ₄	188.104859	203.09166	[M+H] ⁺	1.333	9.2
Oligandriortholactone	C ₂₃ H ₃₀ O ₉	450.188983	451.19693	[M+H] ⁺	1.637	15.4
Ethyl plakortide Z	C ₁₆ H ₃₀ O ₄	286.21440945	269.21128	[M-H ₂ O+H] ⁺	0.598	6.4
alpha-Ketopantoate	C ₇ H ₁₂ O ₃	144.078644241	145.08589	[M+H] ⁺	-0.199	5.7
5-Hydroxycoumarin	C ₉ H ₆ O ₃	162.031694	163.03902	[M+H] ⁺	0.062	3.7
Coumarin	C ₉ H ₆ O ₂	146.036779	147.04399	[M+H] ⁺	-0.451	5.4
Cordytropolone	C ₉ H ₈ O ₄	180.042259	181.04965	[M+H] ⁺	0.649	7.5
2-Furoic acid	C ₅ H ₄ O ₃	112.016044	113.02331	[M+H] ⁺	-0.297	3.6
2-Oxobutyric acid	C ₄ H ₆ O ₃	102.031694	103.03915	[M+H] ⁺	1.775	6.4
2-Acetolactate	C ₅ H ₈ O ₄	132.042259	133.04952	[M+H] ⁺	-0.054	7.9
Citramalic acid	C ₅ H ₈ O ₅	148.037173	149.04435	[M+H] ⁺	-0.650	6.7
Isocitric acid	C ₆ H ₈ O ₇	192.027003	193.03451	[M+H] ⁺	1.120	5.1
3-Hydroxybenzoic acid	C ₇ H ₆ O ₃	138.031694	139.03886	[M+H] ⁺	-0.784	5.2
Cyanomaclurin	C ₁₅ H ₁₂ O ₆	288.063388	289.07099	[M+H] ⁺	1.119	14.6
n-Butyl-beta-D-fructopyranoside	C ₁₀ H ₂₀ O ₆	236.125988	237.13373	[M+H] ⁺	1.975	10.5
Lucidin 3-O-primeveroside	C ₂₆ H ₂₈ O ₁₄	564.147906	565.15595	[M+H] ⁺	1.344	13.9
Conduritol A	C ₆ H ₁₀ O ₄	146.057909	147.06517	[M+H] ⁺	-0.130	7.0
Cnidioside A	C ₁₇ H ₂₀ O ₉	368.110732	369.11847	[M+H] ⁺	1.280	1.1
Pyruvic acid	C ₃ H ₄ O ₃	88.016044	89.02343	[M+H] ⁺	1.403	1.2
Tagitinin A	C ₁₉ H ₂₈ O ₇	368.183503	369.19109	[M+H] ⁺	0.849	20.7
L-Dehydroascorbate	C ₆ H ₅ O ₆	173.008613	157.01342	[M-H ₂ O+H] ⁺	1.559	10.2

2-Ketoisovalerate	C ₅ H ₈ O ₃	116.047344	117.05463	[M+H] ⁺	0.075	4.8
Nodosin	C ₂₇ H ₃₀ O ₁₅	594.15847025	595.16653	[M+H] ⁺	1.362	2.1
Itaconic acid	C ₅ H ₆ O ₄	130.026609	131.02662	[M+H] ⁺	0.122	11.3
Penitricin D	C ₄ H ₄ O ₂	84.021129	85.02836	[M+H] ⁺	-0.432	7.54
Suberic acid	C ₈ H ₁₄ O ₄	174.089209	175.09663	[M+H] ⁺	0.987	7.94
2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	C ₆ H ₈ O ₄	144.042252	145.04945	[M+H] ⁺	-0.583	-
Clandonensine	C ₁₂ H ₂₀ O ₇	276.120903	277.12828	[M+H] ⁺	0.379	14.5
3beta-Hydroxy-5alpha-pregn-16-en-20-one	C ₂₁ H ₃₂ O ₂	316.24023	299.23712	[M-H ₂ O+H] ⁺	0.788	14.5
Alatol	C ₁₇ H ₁₈ O ₆	318.110338	319.17538	[M+H] ⁺	0.790	17.2
Jioglutin C	C ₁₁ H ₁₈ O ₆	246.110338	233.10232	[M+H] ⁺	1.466	10.6
Phosphate	H ₃ O ₄ P	97.97689557	98.98438	[M+H] ⁺	2.114	-
4-(4'-Hydroxy-3'-methylbutoxy)benzaldehyde	C ₈ H ₈ O ₃	152.047344	231.09944	[M+H] ⁺	1.174	13.0
3R-(3R-hydroxybutyryloxy)butyric acid	C ₈ H ₁₄ O ₅	190.084122	173.08072	[M-H ₂ O+H] ⁺	-0.680	2.2
2-Senecioid-4-vinylphenol	C ₁₃ H ₁₄ O ₂	202.09938	203.10640	[M+H] ⁺	-1.241	10.0
Jioglutolide	C ₉ H ₁₄ O ₄	186.089209	187.09618	[M+H] ⁺	-1.658	8.3
Hydroxypyruvic acid	C ₃ H ₄ O ₄	104.010959	105.01836	[M+H] ⁺	1.164	4.8
Fucoserratene	C ₈ H ₁₂	108.0939	109.10115	[M+H] ⁺	-0.229	2.0
Ononitol	C ₇ H ₁₄ O ₆	194.07904	195.08658	[M+H] ⁺	1.370	9.5
Foliasalacioside B2	C ₂₄ H ₄₀ O ₁₁	504.56800	505.26442	[M+H] ⁺	0.041	7.6
Botrytinone	C ₇ H ₁₀ O ₄	158.057909	159.06514	[M+H] ⁺	-0.419	6.5
Dearabinosyl pneumonanthoside	C ₁₉ H ₃₀ O ₇	370.199154	371.20678	[M+H] ⁺	0.944	21.8
(+)-Cyclophellitol	C ₇ H ₁₂ O ₅	176.068473	177.07603	[M+H] ⁺	1.591	20.7
Laurentiquinone C	C ₁₈ H ₂₂ O ₇	350.136553	452.11836	[M+H] ⁺	0.588	2.8
L-(-)-Malic acid	C ₄ H ₆ O ₅	134.021523	135.02871	[M+H] ⁺	-0.652	7.3
Dracunculifoside B	C ₂₂ H ₂₂ O ₁₂	478.111126	479.11868	[M+H] ⁺	0.370	4.8
Annularin C	C ₁₀ H ₁₄ O ₄	198.089209	215.09173	[M+H] ⁺	1.527	10.0
Integristerone A	C ₂₇ H ₄₄ O ₈	496.303618	497.31151	[M+H] ⁺	1.059	10.5
Calocedimer A	C ₃₀ H ₄₆ O ₂	438.34978071	439.35710	[M+H] ⁺	0.120	4.0
Sarmentol A	C ₁₃ H ₂₆ O ₃	230.1881947	231.19586	[M+H] ⁺	1.472	9.2
Cyclonervilasterol	C ₂₉ H ₄₆ O	410.35486609	411.36226	[M+H] ⁺	0.315	16.3
Yardenone A	C ₃₀ H ₄₈ O ₆	504.345089	505.35252	[M+H] ⁺	0.301	3.5
Dendronesterone B	C ₂₈ H ₄₄ O	396.339216	397.34686	[M+H] ⁺	0.925	29.9
10-Hydroxyaloin B	C ₂₁ H ₂₂ O ₁₀	434.121307	435.12896	[M+H] ⁺	1.211	29.1
3-Methyl-2-butenic acid	C ₅ H ₈ O ₂	100.052429494	101.05977	[M+H] ⁺	0.765	5.9
(-)-Amurensisin	C ₂₂ H ₁₆ O ₁₀	440.074347	441.08202	[M+H] ⁺	0.954	7.3
Oligandrumin B	C ₁₇ H ₂₄ O ₇	340.15220312	341.15969	[M+H] ⁺	0.615	18.2
Hyperxanthone F	C ₁₆ H ₁₂ O ₇	316.05830274	317.05864	[M+H] ⁺	1.077	1.8
Globostelletin	C ₃₀ H ₄₄ O ₄	468.32396	469.33148	[M+H] ⁺	0.851	7.8
Podolactone B	C ₁₉ H ₂₂ O ₉	394.126382	395.13438	[M+H] ⁺	1.825	23.0
2-Hydroxycampholonic acid	C ₁₀ H ₁₆ O ₄	200.104859	201.11232	[M+H] ⁺	0.921	8.4
Succinic anhydride	C ₄ H ₄ O ₃	100.016044	101.02340	[M+H] ⁺	0.772	5.2
Suranone	C ₂₄ H ₃₈ O ₃	374.28209508	375.28937	[M+H] ⁺	-0.017	24.5
Lepidimoide	C ₁₂ H ₁₈ O ₁₀	322.089997	345.07977	[M+H] ⁺	1.792	18.2
Moniliformin	C ₄ H ₂ O ₃	98.000394	99.00773	[M+H] ⁺	0.592	3.6

Didemnimide A	C ₁₅ H ₁₀ N ₄ O ₂	278.080376	279.08697	[M+H] ⁺	2.642	14.1
5-Hydroxy-1-methoxyxanthone	C ₁₄ H ₁₀ O ₄	242.057909	243.06549	[M+H] ⁺	1.262	12.0
Leptoclinidamine B	C ₁₆ H ₁₉ N ₅ O ₅	361.138619	362.14561	[M+H] ⁺	-0.788	20.7
L-Galactono-1,4-lactone	C ₆ H ₁₀ O ₆	178.047738	179.05519	[M+H] ⁺	0.672	8.6
(-)-(1R)-8-Hydroxy-4-p-menthen-3-one	C ₁₀ H ₁₆ O ₂	168.11503	169.12227	[M+H] ⁺	-0.035	6.1
Zinolol	C ₁₄ H ₂₁ NO ₈	331.126717	332.13428	[M+H] ⁺	0.878	16.5
Tuberostemospironine	C ₁₃ H ₁₉ NO ₄	253.131408	254.13877	[M+H] ⁺	0.371	3.9
N-Methylpyrrolidine	C ₅ H ₁₁ N	85.089149	86.09647	[M+H] ⁺	0.486	3.0
2-Oxo-N-acetyldopamine	C ₁₀ H ₁₁ NO ₄	209.068808	210.07646	[M+H] ⁺	1.773	6.3
L-Tryptophan	C ₁₁ H ₁₂ N ₂ O ₂	204.089878	205.09742	[M+H] ⁺	1.277	3.5
6-Hydroxykynurenic acid	C ₁₀ H ₇ NO ₄	205.037508	206.04507	[M+H] ⁺	1.592	3.9
Kaempferide 3-rhamnoside-7-(6''-succinylglucoside)	C ₃₂ H ₃₆ O ₁₈	708.190164	709.19821	[M+H] ⁺	1.106	24.9
YUA 001	C ₁₃ H ₁₉ NO ₂	221	222.14917	[M+H] ⁺	1.426	9.0
2R-Hydroxymethyl-3S-hydroxypyrrolidine	C ₅ H ₁₁ NO ₂	117.078979	118.08615	[M+H] ⁺	-0.882	2.0
Spinamycin	C ₄₃ H ₇₄ N ₂ O ₁₄	842.514005	287.13976	[M+H] ⁺	2.588	12.3
Betalamic acid	C ₉ H ₉ NO ₅	211.048072	212.05570	[M+H] ⁺	1.489	8.8
Pyroglutamic acid	C ₅ H ₇ NO ₃	129.042593	130.04988	[M+H] ⁺	0.077	5.0
Lutessine	C ₁₉ H ₂₁ NO ₆	359.376	360.14477	[M+H] ⁺	1.545	19.4
Guvacoline	C ₇ H ₁₁ NO ₂	141.078979	142.08624	[M+H] ⁺	-0.088	3.5
Salinosporamide H	C ₁₅ H ₂₁ NO ₄	279.147058	280.15456	[M+H] ⁺	0.205	8.3
2-Pyrrolidone	C ₄ H ₇ NO	85.052764	86.06028	[M+H] ⁺	2.752	-
L-Pipecolic acid	C ₆ H ₁₁ NO ₂	129.078979	130.08613	[M+H] ⁺	-1.018	2.7
L-Tyrosine	C ₉ H ₁₁ NO ₃	181.073893	182.08144	[M+H] ⁺	0.987	6.3

Figure 1

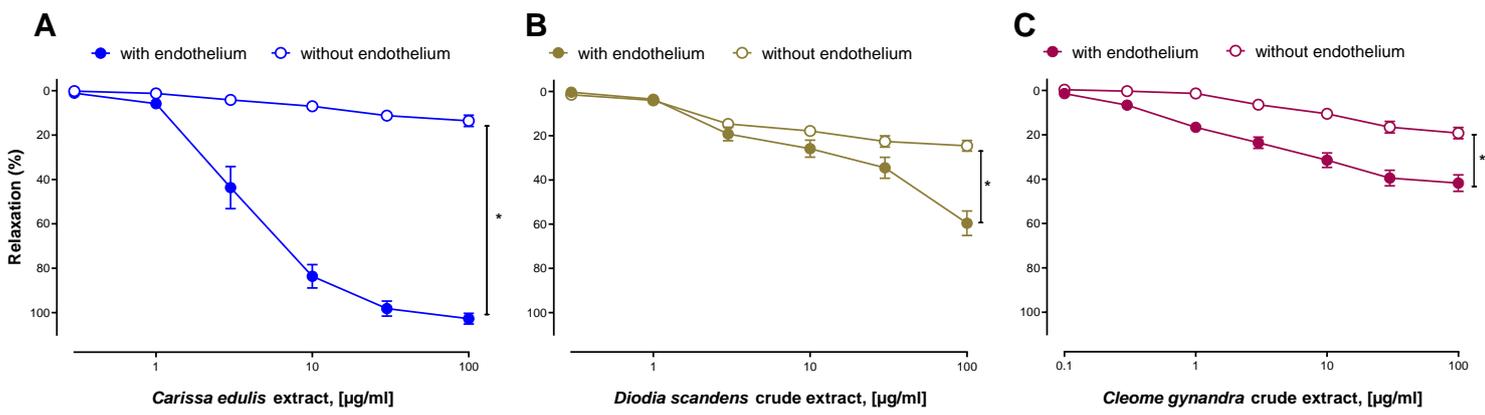


Figure 2

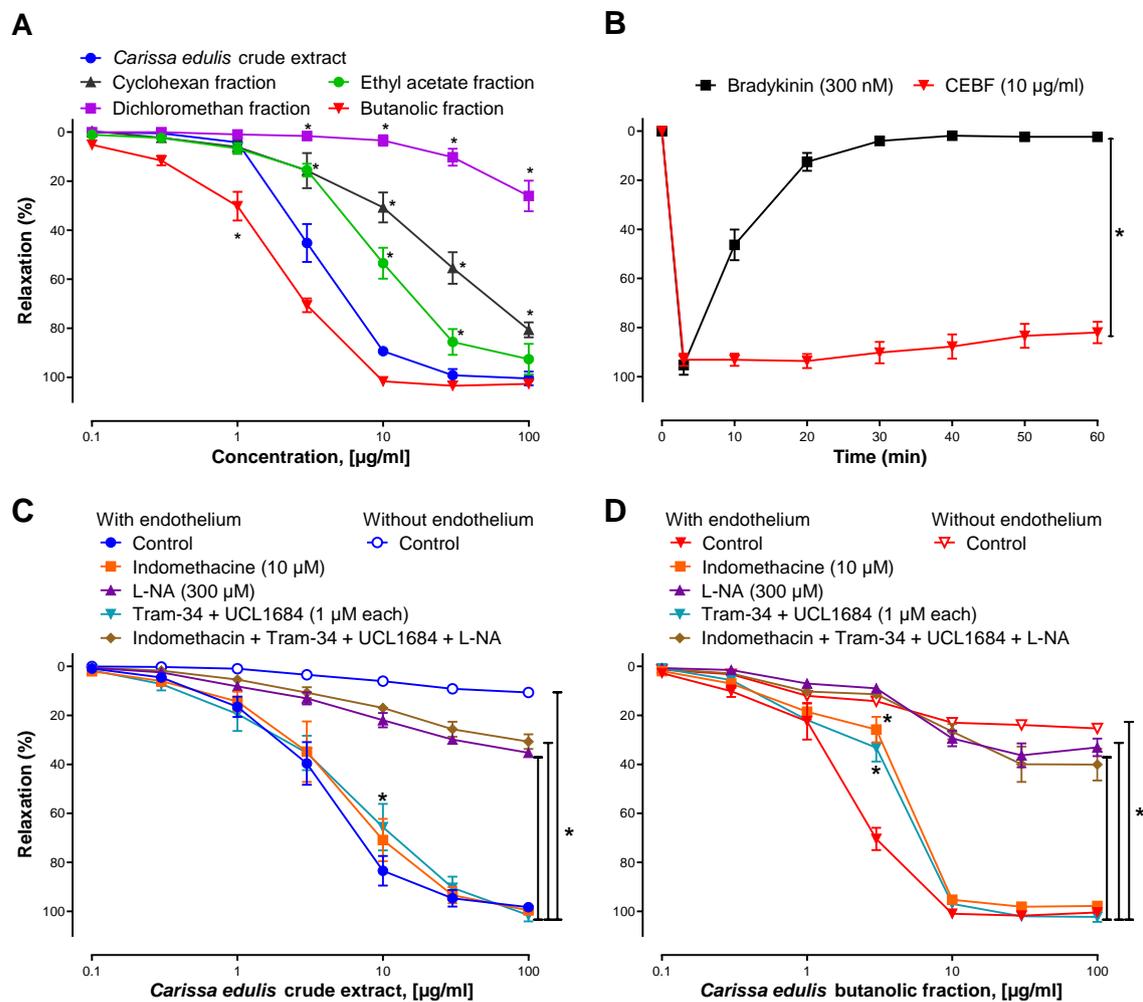


Figure 3

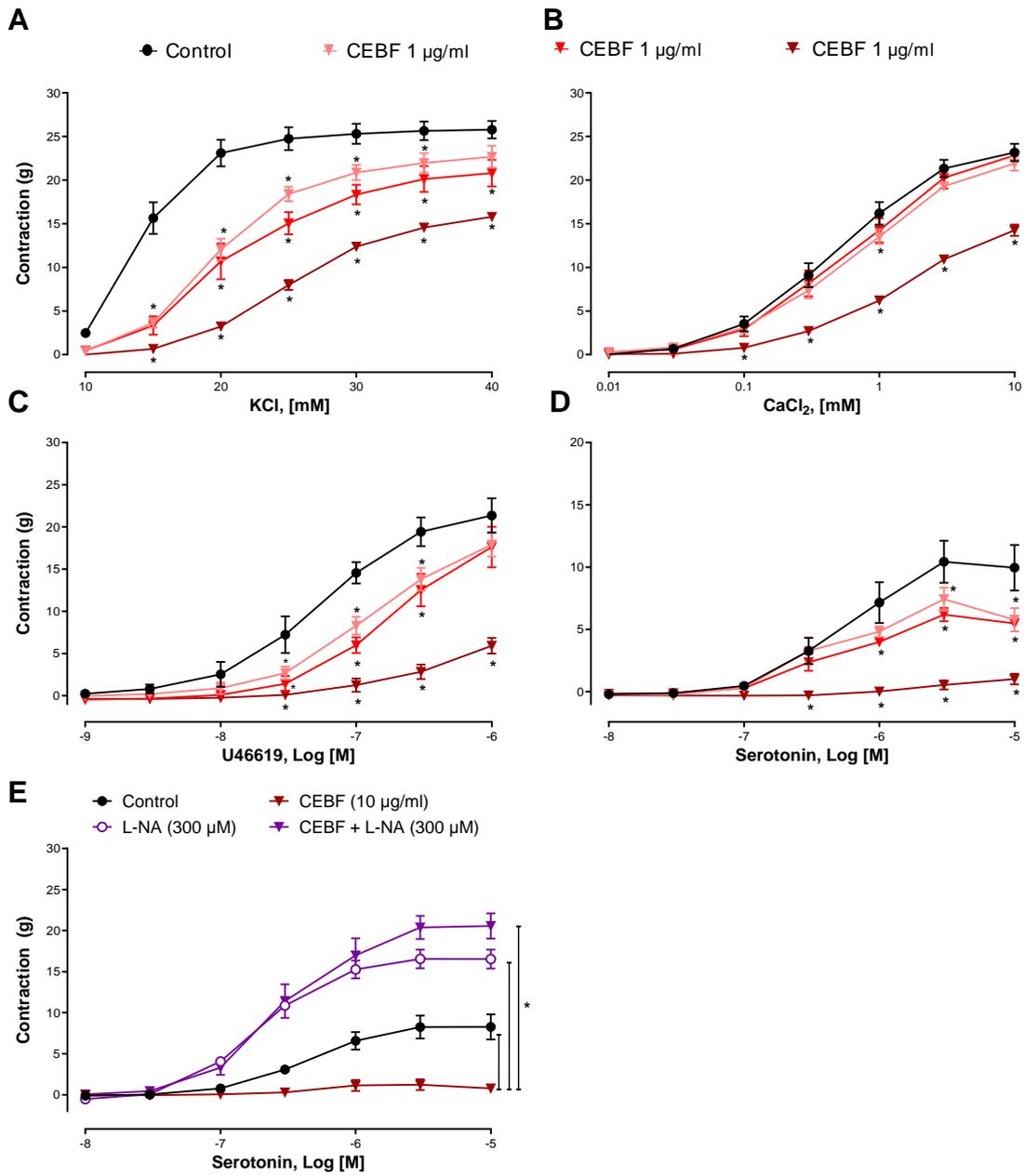


Figure 4

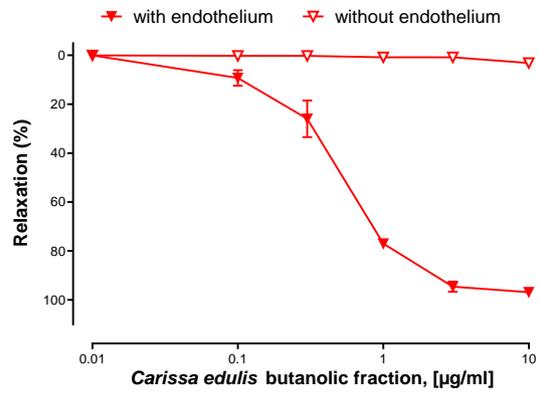


Figure 5

