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Original Article

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

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ABSTRACT

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

Aim: The study investigated the vasoreactivity of the plants and assessed the underlying mechanisms using isolated arteries.

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

Results: The aqueous-ethanolic crude extract of *C. edulis* (CECE) induced potent relaxations that were exclusively endothelium-dependent and more pronounced than those to *D. scandens* and *C. gynandra*. The *n*-butanolic fraction of *C. edulis* (CEBF) was more active than the cyclohexane, dichloromethane, and ethyl acetate fractions. The relaxation induced by CECE and CEBF were inhibited by N^G-nitro-L-arginine and affected neither by TRAM-34 plus UCL 1684 nor by indomethacin. CEBF induced sustained endothelium-dependent relaxations for at least 60 min, and inhibited, in a concentration-dependent manner, contractions to KCl, $CaCl_2$, U46619 and serotonin in rings with endothelium. Analysis of CEBF by LC—HRMS indicated the presence of polyphenols, terpenes, and alkaloids. Exposure of coronary artery and aorta rings to CEBF caused the accumulation of polyphenols predominantly in the endothelium.

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

Abbreviations: $CaCl_2$, Calcium dichloride; CECE, *C. edulis* crude extract; CECF, *C. edulis* cyclohexane fraction; CEDF, *C. edulis* dichloromethane fraction; CEEF, *C. edulis* ethyl acetate fraction; CEBF, *C. edulis* butanolic fraction; EDH, Endothelium-dependent hyperpolarization; EPA:DHA, Eicosapentaenoic acid: docosahexaenoic acid; eNOS, Endothelial NO synthase; IK_{Ca} channels, Intermediate-conductance calcium-activated potassium channels; KCl, Potassium chloride; KH_2PO_4, Potassium dihydrogen phosphate; LC-HRMS, Liquid chromatography-high resolution mass spectrometry; L-NA, N^G-nitro-L-arginine; L-NAME, L-nitro-arginine methyl ester; $MgSO_4$, Magnesium sulfate; MeOH, Methanol; NaCl, Sodium chloride; NaOH, Sodium hydroxide; $NaHCO_3$, Sodium bicarbonate; SK_{Ca} channels, Small conductance calcium-activated potassium channels.

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Introduction

Cardiovascular disease is the most prevalent disease worldwide (Mendis et al., 2007). Although hypertension is a major cardiovascular risk factor identified initially in Western industrialized countries (Maione et al., 2013), its prevalence is increasing continuously in developing countries and, in particular, in sub-Saharan Africa (Tokoudagba et al., 2010). Studies conducted in 2008 in 12 departments of Benin on 6853 people aged 25–64 years old showed that the prevalence of hypertension is 27.9% (Houinato et al., 2012). Additional studies have been carried out in 2015 on 1777 people aged over 25 years in the population of Tanvè, a rural area 150 km north of Cotonou, and on 717 people aged 18–64 years in the Atlantic region of Benin. These studies showed a prevalence of hypertension of 32.9% in the Tanvè population and 33.9% in the Atlantic region (Desormais et al., 2019; Houssou et al., 2020). It is estimated that 60–90% of the general African population uses folk medicine in the management of diseases especially when life-long medication is required such as in the case of hypertension (Balogun and Ashafa, 2019), and also due to the easier availability and the lower cost compared to conventional medicines (Houngbeme et al., 2014; Lawson et al., 2016; Nunes et al., 2015; Sen and Chakraborty, 2017; Yuan et al., 2016). *Carissa edulis* Vahl (*C. edulis*), *Diodia scandens* Sw. (*D. scandens*) and *Cleome gynandra* L. (*C. gynandra*) are three plants traditionally used in Benin for the treatment of hypertension (U. Hounguè, personal communication). *C. edulis* is a perennial thorny shrub of the family of Apocynaceae widespread in Africa, Australia, Vietnam, Yemen and India (Al-Youssef and Hassan, 2014). The biological activities and isolated molecules of *C. edulis* have been recently summarized in our review (Hounguè et al., 2022). *D. scandens* (Rubiaceae) is an evergreen perennial herb, which has an alternate leaf arrangement, (Akpuaka et al., 2018). *C. gynandra* is a plant of the family of Capparidaceae, which grows in most tropical countries. The leaves and seeds of *C. gynandra* have long been used as indigenous medicine for treatment of headaches and stomach aches (Narendhirakannan et al., 2007). The three plants have been reported to have diuretic, analgesic, antioxidant, antimicrobial, antidiabetic, and anticancer activities, and the characterization of their phytochemical composition indicated the presence of flavonoids, phenolic compounds, chlorogenic acid derivatives, lignans, sesquiterpenes, sterols, triterpenes, coumarins, and cardiac glycosides (Chandradevan et al., 2020; Fanta Yadang et al., 2019; Bala et al., 2010; Ibrahim et al., 2010; Tolo et al., 2006; El-Fiky et al., 1996; Akah et al., 1993; Akpuaka et al., 2018; Sango et al., 2016; Ya'u et al., 2013). Acute oral toxicity studies of the leaves and roots of the three plants revealed no sign of toxicity, mortality or reduction of body weight in adult Wistar rats up to 2000 mg/kg for *C. edulis* and *C. gynandra,* and 800 mg/kg for *D. scandens* (Dossou-Yovo et al., 2021; Asiedu-Larbi et al., 2020; Osseni et al., 2017, 2016; Shaik et al., 2013). The possibility that the three plants affect vascular tone that contributes to regulate vascular resistance and, hence, blood pressure, remains poorly studied. Such a hypothesis is supported by the fact that numerous natural products including grape-derived polyphenols and *Crataegus* spp. (Hawthorn) have been shown to cause endothelium-dependent relaxations of isolated arteries by stimulating the endothelial formation of nitric oxide (NO), a potent inhibitor of vascular tone and platelet activation (Schini-Kerth et al., 2010a; Vanhoutte, 2018).

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

Materials and methods

Plant material and extraction

The leaves of *C. edulis, D. scandens* and *C. gynandra* were collected at Togbin, Godomey, Benin in September 2017 and identified by Pr Hounnankpon Yedomonhan, Professor of Botany at the University of Abomey-Calavi, Benin. The reference specimens were deposited at the National Herbarium of the University of Abomey-Calavi, Benin. The samples were dried, powdered (100 g), and macerated at room temperature with ethanol/water (6:4, v/v) (3 × 300 ml for 72 h each). For each plant, the filtered extraction solutions were combined and evaporated at reduced pressure to produce a dry extract with a yield of about 25%, 21% and 23% for *C. edulis, D. scandens* and *C. gynandra,* respectively. The aqueous ethanolic extract of the plant showing the greatest vasorelaxation was successively fractioned using cyclohexane, dichloromethane, ethyl acetate and then butanol.

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

Chemicals and drugs

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

Liquid chromatography coupled to high resolution mass spectrometry analysis

CEBF was analyzed using liquid chromatography coupled to high resolution mass spectrometry (LC—HRMS) on an UltiMate 3000 system (Thermo) coupled to an Impact II (Bruker) quadrupole time-of-flight (Q-TOF) spectrometer. Chromatographic separation was performed on an Acquity UPLC ® BEH C18 column (2.1 × 100 mm, 1.7 µm, Waters) equipped with an Acquity UPLC ® BEH C18 pre-column (2.1 × 5 mm, 1.7 µm, Waters) using a gradient of solvents A (Water, 0.1% formic acid) and B (MeOH, 0.1% formic acid). Chromatography was carried out at 35 °C with a flux of 0.3 ml.min^{-1}, starting with 5% B for 2 min, reaching 100% B at 10 min, holding 100% for 3 min and coming back to the initial condition of 5% B in 2 min, for a total run time of 15 min. Extract was kept at 4 °C, 5 µl were injected in full loop mode with a washing step after sample injection with 150 µl of wash solution (H_2O/MeOH, 90/10, v/v). The spectrometer was equipped with an electrospray ionization (ESI) source and operated in positive ion mode on a mass range from 20 to 1000 Da with a spectra rate of 2 Hz in AutoMS/MS fragmentation mode. The end plate offset was set at 500 V, capillary voltage at 2500 V, nebulizer at 2 Bar, dry gas at 8 lmin^{-1} and dry temperature at 200 °C. The transfer time was set at 20–70 µs and MS/MS collision energy at 80–120% with a timing of 50–50% for both parameters. The MS/MS cycle time was set to 3 s, absolute threshold to 816 cts and active exclusion was used with an exclusion threshold at 3 spectra, release after 1 min and precursor ion was reconsidered if the ratio current intensity/previous intensity was higher than 5. A calibration segment was included at the beginning of the runs allowing the injection of a calibration solution from 0.05 to 0.25 min. The calibration solution used

was a fresh mix of 50 ml isopropanol/water (50/50, v/v), 500 µl NaOH 1 M, 75 µl acetic acid and 25 µl formic acid. The spectrometer was calibrated on the [M+H] + form of reference ions (57 masses from *m/z* 22.9892 to *m/z* 990.9196) in high precision calibration (HPC) mode with a standard deviation below 1 ppm before the injections for each polarity mode, and re-calibration of each raw data was performed after injection using the calibration segment.

Metabolite identification

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

Determination of total phenols content

The total phenols content of CECE and its fractions was determined in triplicate and expressed as gallic acid equivalents (GAE) using the Folin–Ciocalteu reagent (Singleton and Rossi, 1965) that was adapted for microplate reader (Auger et al., 2010). The Folin-Ciocalteu reagent contains a mixture of phosphotungstic and phosphosmolybdic acids, which will react with phenols to form a blue complex visible at 730 nm. In each well of a 96 wells plate, 30 µl of the dilute extract or fraction were deposited, to which 10 µl of Folin-Ciocalteu reagent and 30 µl of Na_2CO_3 (20%) were added. Then, the total volume was adjusted to 200 µl with distilled water. Gallic acid was used as the standard. After 30 min at room temperature, the absorbance was measured at 730 nm using an IEMS Lab System reader, MF spectrophotometer. The calibration curve was constructed using a standard solution of gallic acid (0 to 100 mg/ml). Blank solution: water-ethanol instead of test solution was used. Each assay was carried out in triplicate and expressed as mg/ml gallic acid equivalents.

Vascular reactivity studies

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

Ex vivo polyphenols uptake experiment

Segments of porcine coronary artery and rat thoracic aorta were cleaned of connective tissue and cut into rings (2–3 mm in length). Then, they were washed in Krebs solution and placed in a 24 wells plate containing MCDB131 culture medium (Gibco, ThermoFisher Scientific, Illkirch, France) supplemented with fungizone (250 µg/ml), penicillin (100 UI/ml), streptomycin (100 UI/ml), L-glutamine (2 mM, all from Lonza Levallois-Perret, France). Coronary artery and aorta rings were incubated with CEBF (30, 60, 80 or 100 µg/ml) for 3 min at 37 °C with 5% CO_2. They were then washed twice with PBS before further incubation in PBS containing 0.4% of 2-aminoethyl diphenyl borate for 5 min at 37 °C to determine the uptake of polyphenols according to Jin et al., 2013 with some modifications. Subsequently, rings were washed with cold PBS, snap-frozen in histomolds containing frozen section media (FSC 22, Leica Biosystems, Nanterre, France) in liquid nitrogen. Thereafter, they were cryosectioned at 14 µm and mounted on Superfrost plus slides (ThermoFisher) with Dako medium (fluorescence editing medium, Dako, Agilent Technologies France, Les Ulis, France) and dried for 20 min at room temperature. Slides were then analyzed the same day using a confocal laser scanning microscope (Leica TSC SPE). Quantitative analysis of fluorescence was performed using Image J software (version 1.6 for Windows, NIH).

Statistical analysis

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

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

Name	Molecular formula	Exact Mass (g/mol)	Measured *m/z*	Ion	Δ*m/z* (ppm)	mSigma
Ethyl beta-D-glucopyranoside	$C_8H_{16}O_6$	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	$C_5H_6O_5$	146.021523	129.01819	$[M-H2O+H]^+$	−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	$C_8H_{14}O_2$	142.09938	143.10667	$[M+H]^+$	0.184	4.3
Plumiepoxide	$C_{21}H_{26}O_{13}$	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	$C_9H_{16}O_4$	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	$[M-H2O+H]^+$	0.598	6.4
alpha-Ketopantoate	$C_7H_{12}O_3$	144.078644241	145.08589	$[M+H]^+$	−0.199	5.7
5-Hydroxycoumarin	$C_9H_6O_3$	162.031694	163.03902	$[M+H]^+$	0.062	3.7
Coumarin	$C_9H_6O_2$	146.036779	147.04399	$[M+H]^+$	−0.451	5.4
Cordytropolone	$C_9H_8O_4$	180.042259	181.04965	$[M+H]^+$	0.649	7.5
2-Furoic acid	$C_5H_4O_3$	112.016044	113.02331	$[M+H]^+$	−0.297	3.6
2-Oxobutyric acid	$C_4H_6O_3$	102.031694	103.03915	$[M+H]^+$	1.775	6.4
2-Acetolactate	$C_5H_8O_4$	132.042259	133.04952	$[M+H]^+$	−0.054	7.9
Citramalic acid	$C_5H_8O_5$	148.037173	149.04435	$[M+H]^+$	−0.650	6.7
Isocitric acid	$C_6H_8O_7$	192.027003	193.03451	$[M+H]^+$	1.120	5.1
3-Hydroxybenzoic acid	$C_7H_6O_3$	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	$C_6H_{10}O_4$	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	$C_3H_4O_3$	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	$C_6H_5O_6$	173.008613	157.01342	$[M-H2O+H]^+$	1.559	10.2
2-Ketoisovalerate	$C_5H_8O_3$	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	$C_5H_6O_4$	130.026609	131.02662	$[M+H]^+$	0.122	11.3
Penitricin D	$C_4H_4O_2$	84.021129	85.02836	$[M+H]^+$	−0.432	7.54
Suberic acid	$C_8H_{14}O_4$	174.089209	175.09663	$[M+H]^+$	0.987	7.94
2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	$C_6H_8O_4$	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	$[M-H2O+H]^+$	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_3O_4P	97.97689557	98.98438	$[M+H]^+$	2.114	–
4-(4′-Hydroxy-3′-methylbutoxy) benzaldehyde	$C_8H_8O_3$	152.047344	231.09944	$[M+H]^+$	1.174	13.0
3R-(3R-hydroxybutyryloxy) butyric acid	$C_8H_{14}O_5$	190.084122	173.08072	$[M-H2O+H]^+$	−0.680	2.2
2-Senecioyl-4-vinylphenol	$C_{13}H_{14}O_2$	202.09938	203.10640	$[M+H]^+$	−1.241	10.0
Jioglutolide	$C_9H_{14}O_4$	186.089209	187.09618	$[M+H]^+$	−1.658	8.3
Hydroxypyruvic acid	$C_3H_4O_4$	104.010959	105.01836	$[M+H]^+$	1.164	4.8
Fucoserratene	C_8H_{12}	108.0939	109.10115	$[M+H]^+$	−0.229	2.0
Ononitol	$C_7H_{14}O_6$	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	$C_7H_{10}O_4$	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	$C_7H_{12}O_5$	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	$C_4H_6O_5$	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

(*continued on next page*)

Table 1 (*continued*)

Name	Molecular formula	Exact Mass (g/mol)	Measured *m/z*	Ion	Δ*m/z* (ppm)	mSigma
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-butenoic acid	$C_5H_8O_2$	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	$C_4H_4O_3$	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	$C_4H_2O_3$	98.000394	99.00773	$[M+H]^+$	0.592	3.6
Didemnimide A	$C_{15}H_{10}N_4O_2$	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	$C_{16}H_{19}N_5O_5$	361.138619	362.14561	$[M+H]^+$	−0.788	20.7
L-Galactono-1,4-lactone	$C_6H_{10}O_6$	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	$C_5H_{11}N$	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	$C_{11}H_{12}N_2O_2$	204.089878	205.09742	$[M+H]^+$	1.277	3.5
6-Hydroxykynurenic acid	$C_{10}H_7NO_4$	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	$C_5H_{11}NO_2$	117.078979	118.08615	$[M+H]^+$	−0.882	2.0
Spinamycin	$C_{43}H_{74}N_2O_{14}$	842.514005	287.13976	$[M+H]^+$	2.588	12.3
Betalamic acid	$C_9H_9NO_5$	211.048072	212.05570	$[M+H]^+$	1.489	8.8
Pyroglutamic acid	$C_5H_7NO_3$	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	$C_7H_{11}NO_2$	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_4H_7NO	85.052764	86.06028	$[M+H]+$	2.752	–
L-Pipecolic acid	$C_6H_{11}NO_2$	129.078979	130.08613	$[M+H]^+$	−1.018	2.7
L-Tyrosine	$C_9H_{11}NO_3$	181.073893	182.08144	$[M+H]^+$	0.987	6.3

Results

Total phenols content

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

Identification of CEBF-containing metabolites

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

Vasorelaxant effect on porcine coronary artery rings

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

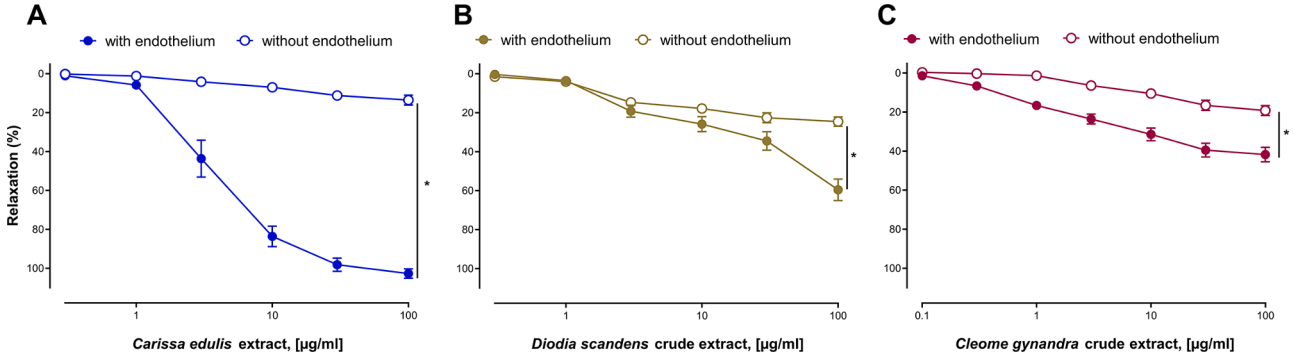

Fig. 1. The crude extract of *C. edulis* **(A)** induced greater endothelium-dependent relaxations than that of *D. scandens* **(B)** and *C. gynandra* **(C)** in porcine coronary artery rings. Results are shown as means ± S.E.M. of 4–6 experiments. *$p < 0.05$ *vs* respective control with endothelium.

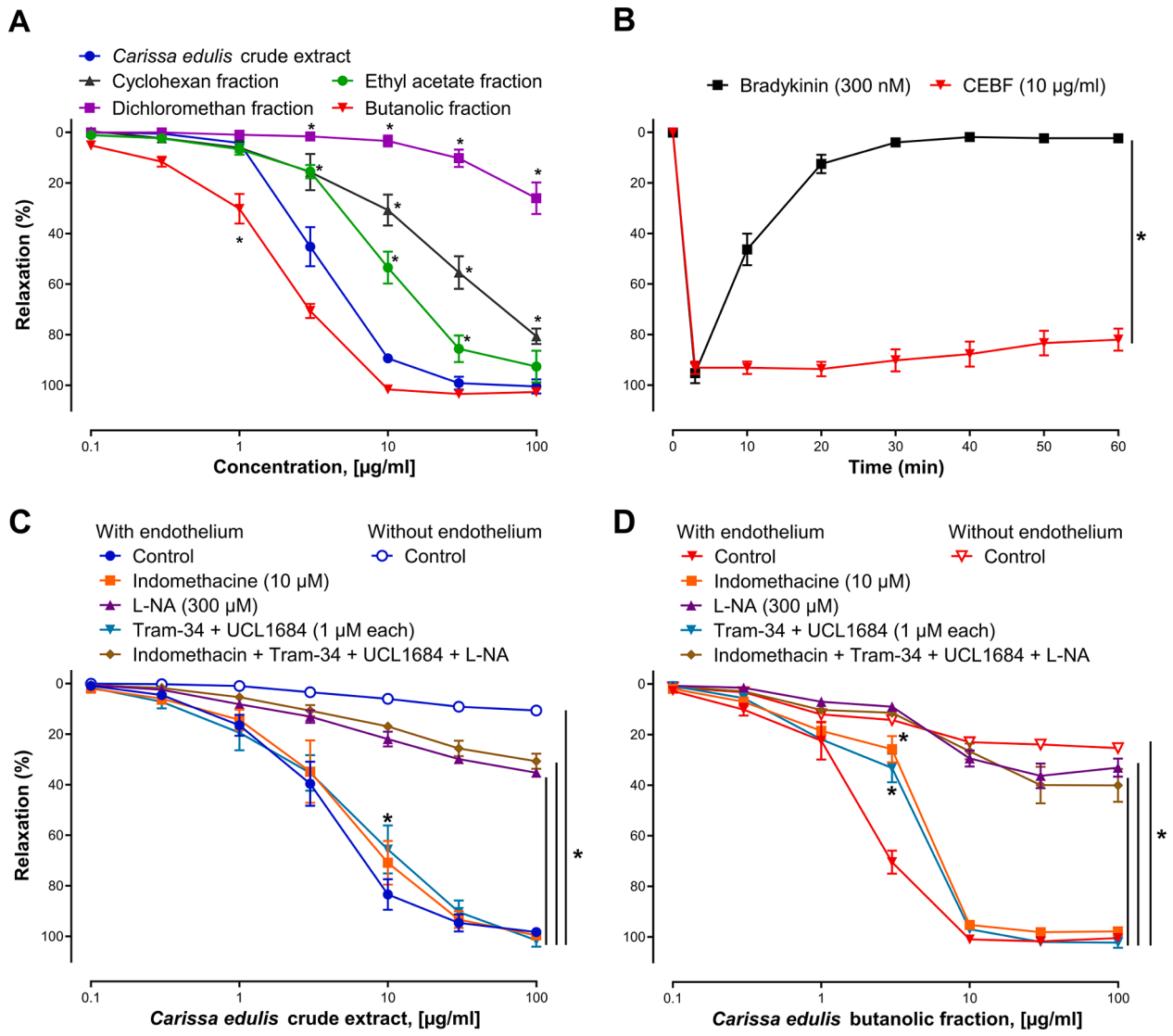

Fig. 2. **(A)** The butanolic fraction of *C. edulis* (CEBF) is more active than the crude extract and the other types of fractions. **(B)** CEBF induced endothelium-dependent relaxations that lasted for at least 60 min whereas those to bradykinin were transient and returned to the pre-contractile level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

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

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

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

CEBF inhibits KCl, $CaCl_2$, U46619 and serotonin-induced contractile responses: role of the endothelial formation of NO

CEBF significantly inhibited the contractile responses of coronary artery rings with endothelium to different vasocontractile agonists including KCl, $CaCl_2$ in the presence of 40 mM of KCl, U46619 and serotonin in a concentration-dependent manner (Fig. 3). At a concentration of 10 µg/ml, CEBF significantly shifted to the right and reduced maximal contractile responses to KCl and $CaCl_2$ whereas those to U46619 and serotonin were markedly reduced (Fig. 3). In the presence of N^G-nitro-L-arginine, contractile responses to serotonin were significantly increased, and the inhibitory effect of CEBF at 10 µg/ml was not observed indicating that NO synthase-derived NO blunted contractile responses.

Vasorelaxant effect on rat aortic rings

Like coronary rings, CEBF induced pronounced relaxations of pre-contracted aortic rings with endothelium compared to those without endothelium in rats (Fig. 4).

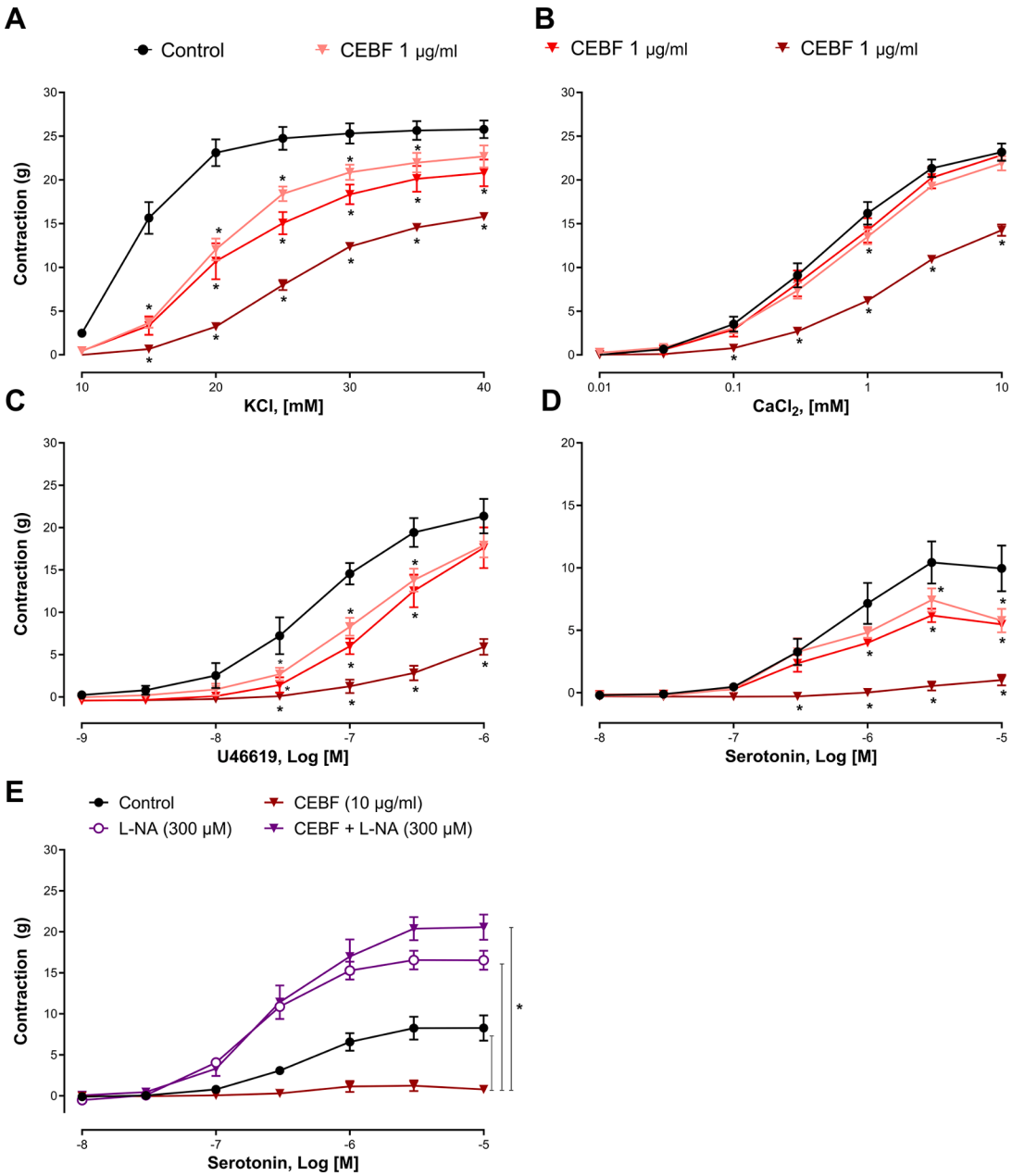

Fig. 3. The butanolic fraction of *C. edulis* inhibits contractile responses to KCl **(A)**, $CaCl_2$ in the presence of 40 mM of KCl **(B)**, U46619 **(C),** and serotonin **(D)** in porcine coronary artery rings with endothelium. Role of endothelium-derived NO was assessed using N^G-Nitro-L-arginine (300 µM) **(E)**. Results are shown as means ± S.E.M. of 4–6 experiments. *$p < 0.05$ *vs* control.

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

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

Discussion

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

Endothelium-dependent relaxations are observed in response to a variety of stimulators including mechanical forces and humoral factors (Vanhoutte, 2018). In most types of conductance arteries, endothelium-dependent relaxations involve mostly NO whereas in

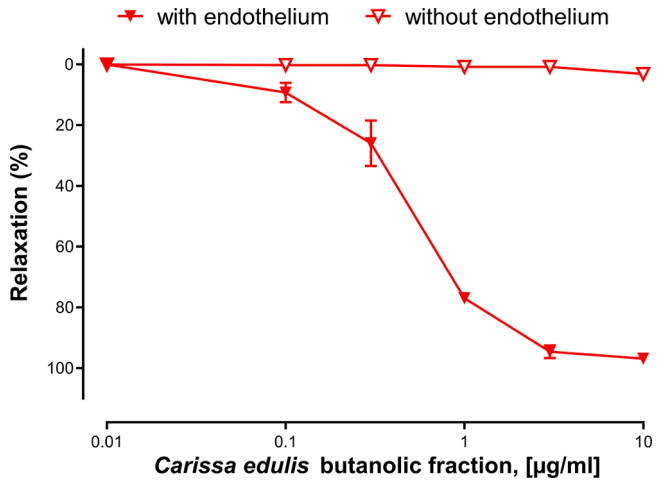

Fig. 4. The butanolic extract of *C. edulis* induced relaxations of rat aortic rings with but not without endothelium. Results are shown as means ± S.E.M. of 4–5 experiments. *$p < 0.05$ *vs* control with endothelium.

resistance arteries and in large coronary arteries NO and EDH are often equally important (Vanhoutte, 2018). Endothelium-derived NO plays a pivotal role in the regulation of vascular homeostasis by regulating vascular tone, and also by inhibiting platelet adhesion and aggregation, and the adhesion of monocytes triggered by a variety of proatherogenic factors (Boo and Jo, 2003; Vanhoutte, 2018). Besides physiological activators, endothelium-dependent relaxations have also been observed in response to natural products such as a polyphenol-rich red wine extract (Ndiaye et al., 2005), an extract of *Dicksonia sellowiana* (Rattmann et al., 2012) and as observed in the present study the polyphenol-rich CECE in porcine coronary arteries and rat aorta. Characterization of the endothelium-dependent relaxations in coronary arteries indicated the involvement of NO and also, to some extent, EDH in response to the red wine extract (Ndiaye et al., 2005) and the *Dicksonia sellowiana* extract (Rattmann et al., 2012). In contrast, the endothelium-dependent relaxation to CECE was exclusively mediated by NO. Of interest, the *C. edulis*-induced endothelium-dependent relaxation is a sustained event in contrast to that induced by the physiological agonist bradykinin, and, hence, might be more appropriate for a persistent control of vascular tone. Natural products have been shown to cause endothelium-dependent relaxations in a variety of blood vessels and species including rat aortic rings (Diebolt et al., 2001), porcine coronary artery rings (Ndiaye et al., 2005), and human internal mammary artery rings (Zgheel et al., 2019). Besides blood vessels, natural products might also be of interest to regulate the tone of the respiratory cartilaginous tube since a hexane extract of *Achillea millefollium* caused relaxations of rat tracheal rings by a non-competitive antagonism of muscarinic receptors, calcium channel blockade, and release of NO (Arias-Durán et al., 2020). In addition to regulation of vascular tone, *C. edulis* has been shown to have antioxidant and anti-inflammatory properties (Al-Yousef and Hassan, 2012; Fanta Yadang et al., 2019). Liu et al. studied the effect of *C. edulis* root bark on cyclooxygenase-2 (COX-2) activity and

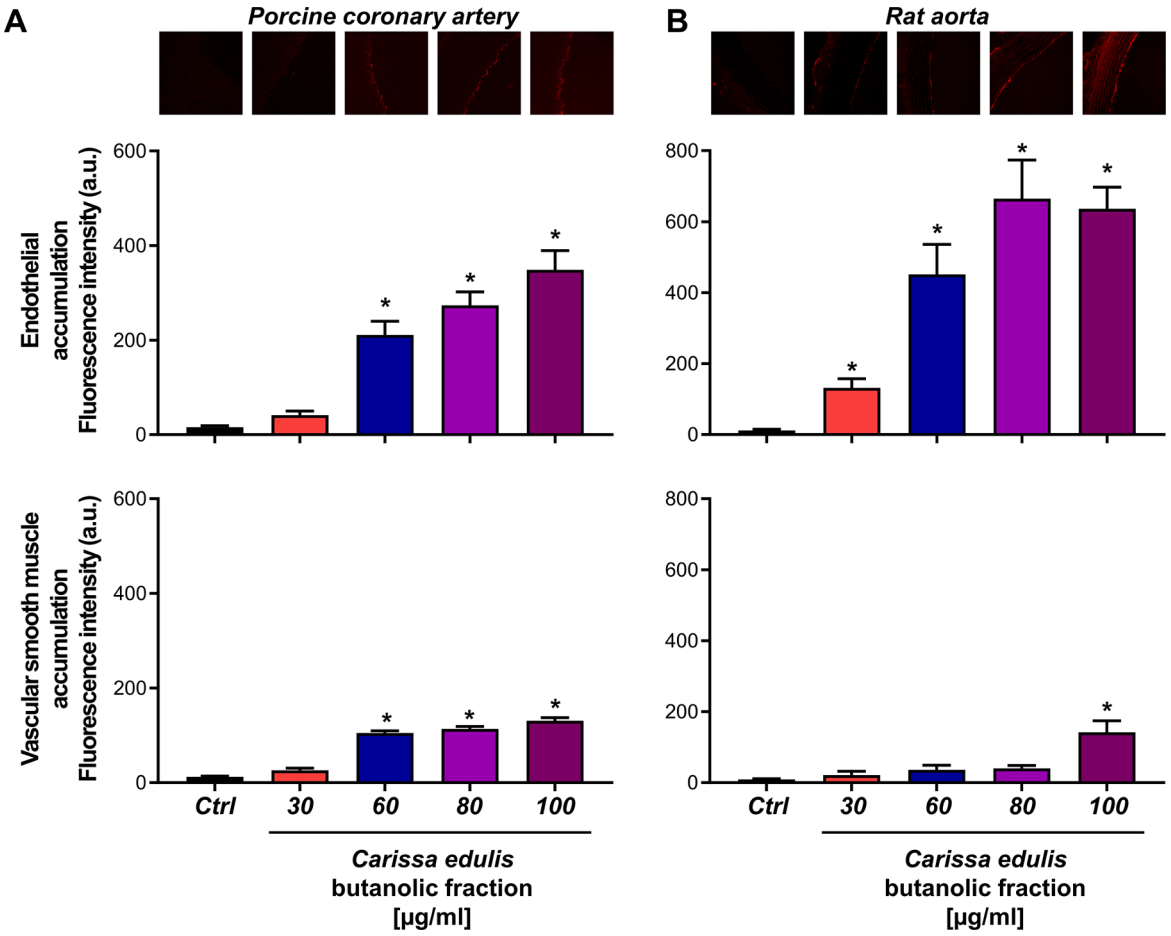

Fig. 5. Exposure of (A) porcine coronary artery rings with endothelium and (B) rat aortic rings with endothelium to the butanolic fraction of *C. edulis* caused the accumulation of polyphenols predominantly in the endothelium in a concentration-dependent manner. Rings were exposed to the butanolic fraction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

observed that the crude extract and four fractions inhibited COX-2 to varying degrees (Liu et al., 2021). Polyphenols have been shown to stimulate endothelial NO synthase to promote the formation of NO, and to up-regulate the expression of endothelial NO synthase in endothelial cells (Schini-Kerth et al., 2010b). Different pathways have been involved in the endothelial formation of NO in response to polyphenols including the redox-sensitive activation of Src/phosphatidylinositol 3-kinase/Akt pathway, the calcium/calmodulin pathway, the activation of estrogen receptors, the 5′ adenosine monophosphate-activated protein kinase and sirtuin 1/Krüppel-like Factor 2 pathways (Anter et al., 2004; Oak et al., 2018).

Contractile responses of blood vessels to KCl are mediated predominantly by membrane depolarization and the subsequent influx of calcium through voltage-operated calcium channels (Kuriyama et al., 1998). In case of G-protein coupled membrane receptor-mediated contractile responses, the activator calcium signal involves both extracellular Ca^{2+} influx through receptor-operated calcium channels and voltage-dependent calcium channels, and also the release of Ca^{2+} from intracellular Ca^{2+} stores (Broekaert and Godfraind, 1979). CEBF inhibited, in a concentration-dependent, manner contractile responses in rings with endothelium induced by either KCl, $CaCl_2$, U46619 or serotonin indicating that the inhibitory effect of CEBF is effective against several signal transduction pathways leading to the Ca^{2+}-dependent activation of contractile proteins. A similar effect has also been observed previously in coronary artery rings in response to the butanolic fraction of a *Berberis orthobotrys* root extract (Alamgeer et al., 2016). CEBF inhibited very effectively contractile responses to U46619 and serotonin. The inhibitory effect of CEBF on contractile responses to serotonin required the presence of a functional endothelium and is prevented by an inhibitor of NO synthase, implying a major role of the endothelial formation of NO. A previous study has also shown that the omega-3 polyunsaturated fatty acid formulation EPA:DHA 6:1 strongly inhibited serotonin-induced contractions in porcine coronary artery rings mostly through an increased endothelial formation of NO (Zgheel et al., 2019).

Analysis of the CECE and CEBF has indicated that they are rich in polyphenols. Previous studies have reported that polyphenols are the major active ingredients in a wide range of medicinal plants used for vascular protection, and involved in endothelium-dependent relaxations of isolated arteries (Andriambeloson et al., 1997; Diebolt et al., 2001; Ndiaye et al., 2005; Tokoudagba et al., 2010). Plant-derived polyphenols can induce endothelium-dependent relaxations following the redox-sensitive activation of Src kinase resulting in the activation of the PI3-kinase/Akt pathway, and the subsequent phosphorylation of eNOS at the activator site Ser1177 (Anselm et al., 2009). Studies evaluating sixty plants with validated antihypertensive/vasorelaxant activity including *Achillea millefolium* and *Ailanthus excelsa* indicated that the biological activity is mostly due to polyphenols followed by terpenes and alkaloids (Maione et al., 2013). Moreover, the antihypertensive/vasorelaxant effect has been reported to include several mechanisms including inhibition of angiotensin-converting enzyme and calcium channels, and activation of endothelial NO synthase and adenylyl cyclase (Maione et al., 2013). Polyphenols of CEBF include flavonoids, phenolic acids, coumarins and anthraquinones. A previous study indicated that flavonoids present in the ethyl acetate extract of *C. edulis* are mainly in the form of glycosylated derivatives (Al-Youssef and Hassan, 2017). Flavonoids have been reported previously to have vasoactive properties. Indeed, luteolin and quercetin from the buds of *Coreopsis tinctoria* caused relaxation of rat aortic rings (Sun et al., 2013), and flavonoids of *Elsholtzia splendens* relaxed aortic rings in both the presence and absence of the endothelium (Wang et al., 2014). Paredes et al. evaluated the antihypertensive effect of several flavonoid extracts (lemon, grapefruit + bitter orange, and cocoa) using a hypertensive rat model after chronic (6 weeks) infusion of the NO synthase inhibitor, L-NAME. The results showed that flavonoid-rich extracts decreased blood pressure induced by chronic administration of L-NAME in rats (Paredes et al., 2018).

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

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

In conclusion, the present findings indicate that an extract of *C. edulis* leaves is a potent vasorelaxant natural product and inhibitor of contractile responses mostly by stimulating the endothelial formation of NO. The vasorelaxant activity is mainly observed with the *n*-butanolic extract of *C. edulis* and is a persistent effect promoting a sustained control of vascular tone. CEBF is rich in polyphenols, which are active natural compounds of a wide range of medicinal plants used for vascular protection, and also contains terpenes and alkaloids that have been shown to have some vasorelaxant properties. The vasorelaxant effect of *C. edulis* is strictly endothelium dependent and is associated with the accumulation of polyphenols preferentially in the endothelium. Thus, the vasorelaxant activity of *C. edulis* can contribute to explain its traditional use for the treatment of hypertension.

Credit author statement

All data were generated in-house, and no paper mill was used. All authors agree to be accountable for all aspects of work ensuring integrity and accuracy.

CRediT authorship contribution statement

Ursula Hounguè: Investigation, Visualization, Formal analysis, Writing – original draft, Writing – review & editing. **Claire Villette:** Investigation, Visualization. **Jean-Marie Tokoudagba:** Conceptualization, Methodology. **Ahmed B. Chaker:** Investigation. **Lamia Remila:** Investigation. **Cyril Auger:** Visualization, Supervision, Writing – review & editing. **Dimitri Heintz:** Resources, Supervision. **Fernand A. Gbaguidi:** Conceptualization, Methodology. **Valérie B. Schini-Kerth:** Conceptualization, Methodology, Supervision, Writing – original draft, Writing – review & editing, Funding acquisition, Project administration.

Declaration of Competing Interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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