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Original Article

Caryocar brasiliense induces vasorelaxation through endothelial Ca^{2+}/calmodulin and PI3K/Akt/eNOS-dependent signaling pathways in rats

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ABSTRACT

Caryocar brasiliense Cambess., Caryocaraceae (pequi) is a typical Brazilian Cerrado tree. A previous study showed that the butanolic fraction of pequi leaves promotes endothelium-dependent relaxation mediated by nitric oxide and that it causes reversible hypotension in rats. In the present study, we investigated the cell signaling pathways associated with the butanolic fraction-induced nitric oxide release, and we characterized the chemical composition of its fraction. Vascular reactivity tests, a western blotting analysis, and a chemiluminescence assay were used to investigate the signaling pathways involved in the vasorelaxant effect of the butanolic fraction. Electrospray Ionization Fourier Transform Ion Cyclotron Resonance Mass Spectrometry was used to characterize the butanolic fraction chemical composition. Vasorelaxation was mediated through the activation of the calmodulin and phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathways, leading to subsequent endothelial nitric oxide synthase phosphorylation and nitric oxide production, as evidenced by western blotting and chemiluminescence assays, respectively. The chemical characterization of the butanolic fraction revealed the presence of 72 oxygenated compounds, whose molecular formulae are compatible with phenolic compounds, suggesting a potential contribution of these compounds for the butanolic fraction vasorelaxant effect. These findings show that the calmodulin and phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathways are involved in the butanolic fraction-induced endothelial nitric oxide synthase activation and are promoted by polyphenol compounds present in the *C. brasiliense* leaves.

Introduction

Caryocar brasiliense Cambess., known as *pequi*, is a tree that belongs to the Caryocaraceae family and is widely distributed in the Cerrado region of Brazil (Lorenzi, 1998). In folk medicine, the seed fat is used for the treatment of respiratory tract diseases, such as asthma and bronchitis (Brandão et al., 2002). The bark is reported to function as a febrifuge and diuretic, and the leaves and fruits are used in the treatment of respiratory, ophthalmologic, and hepatic disorders (Almeida and Silva, 1994; Mors et al., 2000).

The phytochemical screening of *C. brasiliense* revealed the presence of classes of secondary plant metabolites with therapeutic potential, including flavonoids, triterpenes, saponins, and tannins (Oliveira et al., 1968; Magalhães et al., 1988). Antioxidant activity was associated with the presence of gallic acid, quinic acid, quercetin, and quercetin 3-*O*-arabinose compounds in *C. brasiliense* fruits (Roesler et al., 2008). In addition, leishmanicidal, antibacterial, antioxidant, antigenotoxic, anticlastogenic, and hypotensive

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activities were demonstrated in this species (de Paula-Junior et al., 2006; Khouri et al., 2007; Miranda-Vilela et al., 2008).

In a previous study, we showed that the crude hydroalcoholic extract (CHE) from pequi leaves and derived organic fractions produced a significant vasorelaxant effect in rat thoracic aorta. The butanolic organic fraction (BF) obtained from the CHE presented the highest effect when compared with other fractions (hexane, chloroform, and ethyl acetate), and it was similar to the CHE vasorelaxant effect. Subsequently, it was observed that the vasodilation promoted by the BF is endothelium dependent and involves the stimulation of the nitric oxide (NO)/cyclic guanosine monophosphate (cGMP) pathway. Moreover, the BF evoked significant hypotension in anesthetized rats, which was attributed to the gallic acid and quercetin compounds found in the BF (de Oliveira et al., 2012). Gallic acid and quercetin are effective for enhancing the bioavailability of endothelium-derived nitric oxide, may cause vasodilation and, consequently, hypotension (Li et al., 2012; de Oliveira et al., 2016; Sant'Anna et al., 2017).

Considering our previous findings, the present study aimed to elucidate the mediators involved in the BF-induced endothelial NO synthase (eNOS) activation and to characterize the chemical components present in this fraction.

Material and methods

Chemicals and drugs

Phenylephrine (Phe), acetylcholine (ACh), calmidazolium, wortmannin, 2-[*N*-(2-hydroxyethyl)]-*N*-(4-methoxybenzenesulfonyl)] amino-*N*-(4-chlorocinnamyl)-*N*-methylbenzylamine (KN-93), 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo [3,4-d] pyrimidine (PP2), 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, pepstatin A, E-64, bestatin, leupeptin, aprotinin, sodium orthovanadate, phenylmethanesulfonyl fluoride, and dithiothreitol were purchased from Sigma (St. Louis, MO, USA). Wortmannin and the BF were prepared as stock solutions in dimethyl sulfoxide (DMSO), and the other drugs were dissolved in distilled water. The final concentration of DMSO in the bath never surpassed 0.1%.

Plant material and preparation of the BF

The herbal material and procedures used to prepare the BF were previously described (de Oliveira et al., 2012). *Caryocar brasiliense* Cambess., Caryocaraceae, leaves were collected in September 2010, in Gurupi City, State of Tocantins, Brazil. The plant was authenticated by Aristônio Magalhães Teles, and a voucher specimen was deposited in the herbarium of the Institute of Biological Sciences, Federal University of Goiás, under number 1353. The BF was obtained through the sequential extraction process of crude hydroalcoholic extract of *C. brasiliense* leaves (10 g) using hexane, chloroform, ethyl acetate, and *n*-butanol solvents. The remaining *n*-butanol fraction (BF) was evaporated to dryness, and it obtained a yield of 13.7% (w/w).

Animals

Male Wistar rats, 10–12 weeks of age, from the colony of the Federal University of Goiás, were used in this study. The rats were maintained on a 12 h light/dark cycle, under a controlled temperature ($22 \pm 1\,^{\circ}C$), with ad libitum access to food and water. The experimental protocols were performed in accordance with the Ethical Principles in Animal Experimentation adopted by the Brazilian College of Animal Experimentation (COBEA) and were approved by the local Ethics in Research Committee (Protocol CEP/UFG 22/2011).

Preparation of thoracic aorta rings

Vascular reactivity was performed in an organ bath setting, as previously described (de Oliveira et al., 2012). After euthanasia, the thoracic aorta was quickly removed and cleaned in a physiological solution, containing (in mmol) 130 NaCl, 14.9 $NaHCO_3$, 4.7 KCl, 1.18 KH_2PO_4, 1.17 $MgSO_4{\cdot}7H_2O$, 1.6 $CaCl_2{\cdot}2H_2O$, and 5.5 glucose. The aorta was cut into 4-mm rings, which were then mounted in a muscle bath containing a Krebs solution at 37 °C and bubbled with 95% O_2–5% CO_2. Isometric force generation was recorded with an isometric force transducer (AQCAD, AVS Projects, Sao Carlos). A resting tension of 1.5 g was imposed on each ring, and the rings were allowed to equilibrate for 1 h. Endothelium integrity was assessed by measuring the dilatory response to ACh (10 μM) in the Phe contracted vessels (1 μM). For studies of endothelium-intact vessels, a ring was discarded if the relaxation with Ach (10 μM) was not 90% or greater. After assessing the presence of functional endothelium, vascular tissues were allowed to recuperate for at least 1 h, during which, the Krebs–Henseleit solution was replaced every 15 min, before any experiment protocol.

Vascular functional studies

Cumulative concentration–response curves to the BF (from 0.1 to 30 μg/ml) or vehicle were constructed for the aortic rings precontracted with Phe (1 μM). To verify the involvement of the Ca^{2+}/CaM complex, the cumulative concentration–effect curves to the BF were constructed for endothelium-intact aortic rings in the presence of the selective calmodulin inhibitor (calmidazolium, 10 μM). To investigate whether the BF-induced vasodilatation is mediated by a calmodulin-dependent protein kinase II, cumulative concentration–effect curves to the BF were constructed in endothelium-intact aortic rings in the presence of KN-93, a calmodulin-dependent protein kinase II inhibitor (10 μM). To verify the role of the Src kinase and phosphatidylinositol 3-kinase (PI3K), cumulative concentration–effect curves to the BF were constructed in endothelium-intact aortic rings in the presence of either PP2 (a Src kinase inhibitor, 10 μM) or wortmannin (a PI3K inhibitor, 100 nM). In all of the experiments, the rings were exposed to an inhibitor for 30 min before Phe was added.

Western blotting analysis

Endothelium-intact aortic segments incubated with the BF (0.3, 3, and 30 μg/ml) or vehicle for 10 min were subsequently frozen in liquid nitrogen and homogenized in a buffer ([4-{2-aminoetil} benzenesulfonyl fluoride, pepstatin, E-64, bestatin, leupeptin, aprotinin, sodium orthovanadate, PMSF, and sodium fluoride). The proteins were extracted (70 μg), separated by electrophoresis on 10% polyacrylamid gels, and transferred onto nitrocellulose membranes. Non-specific binding sites were blocked with 5% BSA in TBS containing 0.1% Tween 20 (for 1 h at 24 °C). The membranes were incubated with antibodies (at the indicated dilutions) overnight at 4 °C. The antibodies were used as follows: anti-phospho-eNOS (Ser^{1177}) (1:500 dilution; Cell Signaling Technology), anti-Akt (1:1000 dilution; Cell Signaling), anti-phospho-Akt (Ser^{473}) (1:500 dilution; Sigma), anti-PI3K (1:500 dilution; Cell Signaling), anti-phospho-PI3K (Tyr^{458}) (1:500 dilution; Cell Signaling), anti-phospho c-Src (Try^{419}) (1:200 dilution; Santa Cruz), and anti-β-actin (1:2000 dilution; Cell Signaling). After incubation with the secondary antibodies, signals were obtained by chemiluminescence, visualized by autoradiography, and quantified densitometrically (Image J, National Institute of Mental Health, Bethesda, MD). The values for phospho-eNOS (Ser^{1177}) and phospho c-Src (Try^{419}) were normalized to β-actin, and the values for p-PI3K (Tyr^{458}) and for p-Akt (Ser^{473}) were normalized with PI3K

and Akt, respectively. The protein levels were expressed as units relative to the control.

Evaluation of NO metabolite (nitrite and nitrate) production in the presence of the BF

Segments of aortic rings were collected in tubes containing a 20 mM Tris/HCl buffer (pH = 7.4). After centrifugation at 5000 × *g* for 10 min, the supernatant was removed and stored at −20 °C. The NO was determined by the evaluation of its oxidation products content (NOx) (nitrites and nitrates) via an ozone-based chemiluminescence assay. Samples were treated with a 2:1 volume of ice-cold ethanol (ethanol/sample) and centrifuged at 10,000 × *g* for 5 min. The NOx was measured by injecting 5 μl of the supernatant into a glass purge vessel containing vanadium in 1 N HCl, which reduces NOx to NO gas. A nitrogen stream was bubbled through the purge vessel containing vanadium, then through 1 N NaOH, and then into a NO analyzer (Sievers® Nitric Oxide Analyzer 280, GE Analytical Instruments, Boulder, CO, USA), which detects the NO released from the NOx for chemiluminescent detection. The samples were normalized by protein levels. An estimation of the protein concentration was determined by the Bradford protein assay (Bradford, 1976).

Electrospray Ionization Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (ESI FT-ICR MS)

The ultra-high resolution mass spectrum provided by FT-ICR MS enables us to assign molecular formulae to the *m/z* values detected with an error lower than 1 ppm (Ferreira et al., 2014). The mass spectrometer (model 9.4 T Solarix, Bruker Daltonics, Bremen, Germany) was set to operate in the negative ion mode, ESI(−), over a mass range of *m/z* 200–2000. The parameters of the ESI(−) source were as follows: a nebulizer gas pressure of 0.5–1.0 bar, capillary voltage of 3–3.5 kV, and transfer capillary temperature of 250 °C. The mass spectrum was processed using the Compass Data Analysis software package (Bruker Daltonics, Bremen, Germany). A resolving power ≅500,000 and a mass accuracy of <1 ppm provided the unambiguous molecular formula assignments for singly charged molecular ions. The elemental compositions of the compounds were determined by measuring the *m/z* values. The aromaticity level of each molecule can be deduced directly from its DBE (double bond equivalent).

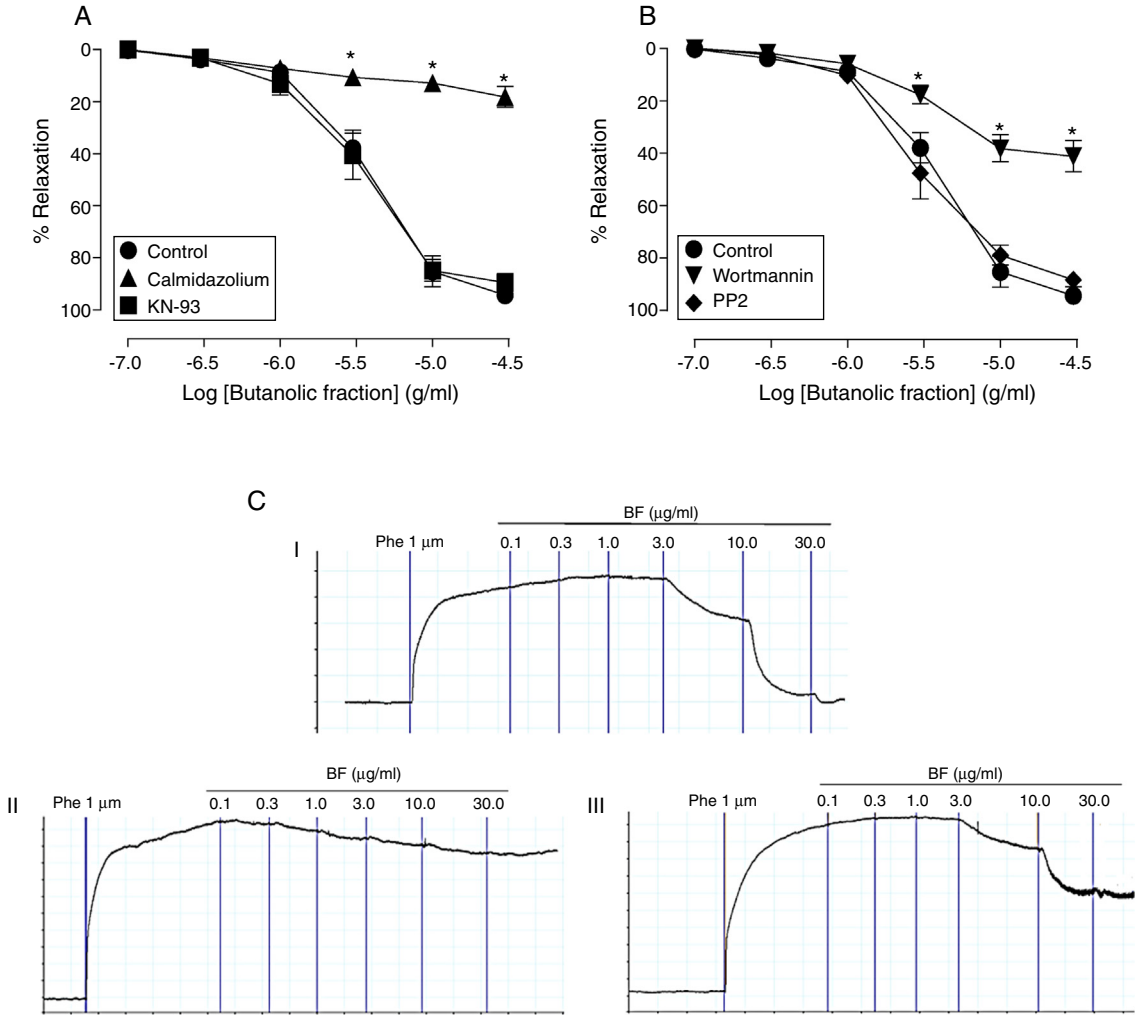


Fig. 1. Cumulative concentration–response curves to the butanolic fraction (BF) of pequi leaves (*Caryocar brasiliense*) were performed in endothelium-intact aortic rings contracted with phenylephrine (1 μM) in the presence of calmidazolium (CaM inhibitor, 10 μM) or KN-93 (CaMKII inhibitor, 10 μM) (A) and in the presence of wortmannin (Pl3-kinase inhibitor, 100 nM) or PP2 (Src kinase inhibitor, 10 μM) (B). (C) Original trace of vasodilation induced by cumulative concentrations of BF (0.1–30.0 μg/ml), as follows: (I) BF in the presence of the vehicle (DMSO 0.1%; control), (II) BF in the presence of calmidazolium, and (III) BF in the presence of wortmannin. Data are expressed as the mean ± SEM of 5–7 experiments performed on preparations obtained from different animals. $^{*}p < 0.05$ when compared to the control (ANOVA and post hoc test Tukey–Kramer).

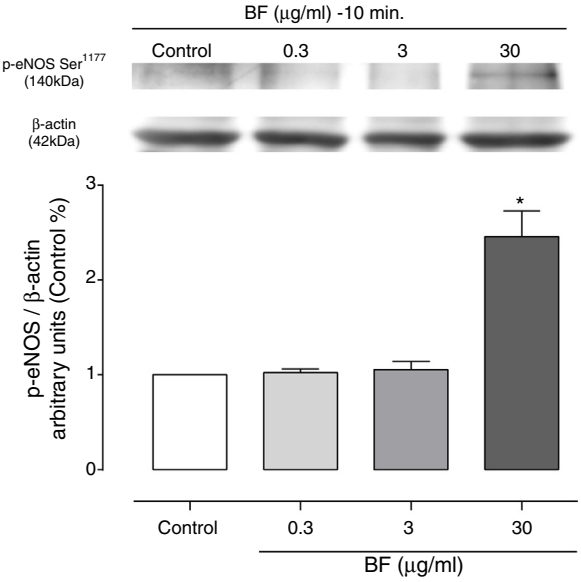


Fig. 2. Butanolic fraction (BF) of pequi leaves (*Caryocar brasiliense*) induces eNOS activation. The effects of the butanolic fraction (BF) of pequi leaves BF (0.3, 3, and 30 μg/ml) on eNOS phosphorylation at Ser^{1177} were determined in endothelium-intact rat thoracic aorta rings. Data are expressed as the means ± SEM of 4–5 different immunoblots (each one corresponding to different animals), and bar graphs are representative of the relative expression of p-eNOS protein normalized to β-actin. $^{*}p < 0.05$ versus control (ANOVA and post hoc test Tukey–Kramer).

Statistical analysis

All values are given as mean ± S.E.M., and *n* denotes the number of animals used in each experiment. The statistical significance was analyzed by using either Student's *t*-test or one-way ANOVA, followed by the Tukey–Kramer post hoc test for comparison between groups. A value of $p < 0.05$ was considered statistically significant. In vascular reactivity experiments, relaxations were expressed as the percentage of Phe-induced contraction. Analyses were performed using a GraphPad Prism version 5.00 for Windows (San Diego, CA, USA).

Results and discussion

Signaling pathways involved in eNOS activation by BF

In order to determine the signaling pathways involved in the BF-induced activation of NO/GMPc signaling, aortic rings were incubated with selective inhibitors prior to the BF addition. The incubation of aortic rings with calmidazolium [a calmodulin (CaM) inhibitor] reduced the BF-induced relaxation ($E_{max} = 14.9 \pm 2.8$) when compared to BF relaxation in the presence of a vehicle ($E_{max} = 94.9 \pm 1.9$). On the other hand, incubation with KN-93 [a CaM dependent protein kinase II (CaMKII) inhibitor] did not significantly alter BF relaxation (Fig. 1A). Treatment of aortic preparations with wortmannin [a PI3K inhibitor] produced a significant inhibition of the relaxing responses to the BF ($E_{max} = 36.1 \pm 4.0\%$), and the addition of PP2 (a Src kinase inhibitor) did not alter the BF-induced relaxation when compared with BF relaxation in the presence of a vehicle (Fig. 1B).

eNOS plays a critical role in maintaining vascular homeostasis, and it is constitutively expressed in the endothelium as the primary source of NO at physiological conditions (Shaul, 2002; Forstermann and Munzel, 2006). NO is produced in the endothelium through the functional eNOS, which oxidizes its substrate L-arginine to L-citrulline and NO. In endothelial cells, the main signal transduction pathway of agonist-stimulated eNOS activation depends on the calcium/calmodulin (Ca^{2+}/CaM) complex. Upon an increase in intracellular Ca^{2+} concentration, Ca^{2+}/CaM binds to the canonical CaM-binding domain in eNOS and increases its catalytic activity (Stuehr, 1997; Fleming and Busse, 1999, 2003). Our results demonstrate that the effect produced by the BF is attenuated in the presence of calmidazolium, showing that calmodulin is involved in the BF vasorelaxant effect.

NO synthesis is also regulated by eNOS phosphorylation, a mechanism known to be Ca^{2+}-independent. eNOS phosphorylation is induced by agents such as insulin, estrogens, and shear stress, which activate protein serine/threonine kinases, such as CaMKII (Abraham et al., 1997; Stuehr, 1997; Fleming and Busse, 2003) and PI3K/Akt (Fulton et al., 1999; Mount et al., 2007) induce eNOS phosphorylation in the serine amino acid at position 1177 (Ser^{1177}) (Dimmeler et al., 1999). The results showed that CaMKII is not involved in the vasorelaxant effect by BF. However, BF-induced relaxation was significantly attenuated by a PI3K inhibitor, confirming the participation of PI3K signaling in the BF vasorelaxant effect. In addition, the results indicate that the PI3K is not activated by Src kinases.

Taken together, these findings suggest that the BF of the crude hydroalcoholic extract of *C. brasiliense* leaves promotes NO production in rat thoracic aorta, by mechanisms involving the CaM-dependent complex and PI3K/Akt signaling.

Characterization of NO production evoked by the BF, via the PI3K/Akt signaling pathway

The BF (30 μg/ml) increased eNOS phosphorylation at Ser^{1177} in rat aortic preparations (Fig. 2), indicating an activation of eNOS. The participation of the PI3K/Akt signaling pathway in the BF-induced eNOS phosphorylation was confirmed by our findings: Akt phosphorylation at Ser^{473} was significantly increased in aortic samples incubated with BF (0.3, 3, and 30 μg/ml, Fig. 3A).

Considering that PI3K is a putative upstream activator of Akt, in the next step of the study, the PI3K phosphorylation levels were evaluated in the presence of BF. As shown in Fig. 3B, the level of phospho-PI3K was significantly increased by the BF. On the other hand, the phosphorylation of Src kinase was not altered in the presence of BF (Fig. 3C), indicating that PI3K activation does not involve Src proteins. Based on these results, it was demonstrated that BF evokes phosphorylation and the stimulation of eNOS via the PI3K–Akt pathway.

The BF-induced NO production was also evaluated. The results showed that levels of NO metabolites were significantly higher in vessels treated with BF 30 μg/ml (4.21 ± 0.43 μM/mg protein) when compared with those in BF absence (2.36 ± 0.26 μM/mg protein) ($p < 0.05$; Fig. 4).

These results clarify the mechanisms by which the BF promotes vasorelaxation. The BF of the crude hydroalcoholic extract of *C. brasiliense* leaves activates the PI3K/Akt signaling pathway, which in turn, activates eNOS by Ser^{1177} phosphorylation. The end point of this cellular signaling sequence is the synthesis of NO, which was confirmed by increased NO metabolite (NOx) levels in rat aorta incubated with the BF.

Chemical characterization of the BF

Fig. 5 illustrates the ESI FT-ICR MS of the BF. The main polar constituents of the BF (sugar derivatives and phenolic compounds) are detected in the deprotonated form $[M–H]^-$ and as a nitrate adduct $[M+NO3^-]^-$. Table 1 shows the measured and theoretical *m/z* values, mass errors, molecular formulas, and DBE of the 72 compounds identified in the BF.

Table 1
Assigned molecular formulae for the measured *m*/*z* values from ESI FT-ICR MS of the butanolic fraction (BF) of *Caryocar brasiliense* leaves.

Measured *m*/*z*	Formula	Theoretical *m*/*z*	Error (ppm)	DBE
232.00987	$[C_7H_6O_5 + NO_3^-]^-$	232.00989	−0.0834	5
242.05164	$[C_6H_6O_5 + NO_3^-]^-$	242.051755	−0.4725	4
254.051751	$[C_7H_{12}O_6 + NO_3^-]^-$	254.051755	−0.0127	2
289.071772	$[C_{15}H_{14}O_6 - H]^-$	289.071762	0.0373	9
291.01468	$[C_{13}H_8O_8 - H]^-$	291.014641	0.1363	10
300.998981	$[C_{14}H_6O_8 - H]^-$	300.998991	−0.0311	12
325.056532	$[C_{14}H_{14}O_9 - H]^-$	325.056506	0.0815	8
331.067064	$[C_{13}H_{16}O_{10} - H]^-$	331.06707	−0.0205	6
343.067052	$[C_{14}H_{16}O_{10} - H]^-$	343.06707	−0.0544	6
352.067443	$[C_{15}H_{14}O_6 + NO_3^-]^-$	352.067405	0.1103	9
353.108958	$[C_{13}H_{22}O_{11} - H]^-$	353.108935	0.065	3
357.082812	$[C_{15}H_{18}O_{10} - H]^-$	357.08272	0.2579	6
361.07769	$[C_{14}H_{18}O_{11} - H]^-$	361.077635	0.1513	6
368.06233	$[C_{15}H_{14}O_7 + NO_3^-]^-$	368.062319	0.0294	9
371.11953	$[C_{13}H_{24}O_{12} - H]^-$	371.1195	0.0818	2
383.062022	$[C_{16}H_{16}O_{11} - H]^-$	383.061985	0.098	9
388.052213	$[C_{14}H_{14}O_9 + NO_3^-]^-$	388.052148	0.1652	8
392.083534	$[C_{14}H_{18}O_9 + NO_3^-]^-$	392.083449	0.2177	6
394.062765	$[C_{13}H_{16}O_{10} + NO_3^-]^-$	394.062713	0.1317	6
404.104645	$[C_{12}H_{22}O_{11} + NO_3^-]^-$	404.104578	0.166	2
406.062595	$[C_{14}H_{16}O_{10} + NO_3^-]^-$	406.062713	−0.292	7
411.093389	$[C_{18}H_{20}O_{11} - H]^-$	411.093285	0.2531	9
420.078486	$[C_{15}H_{18}O_{10} + NO_3^-]^-$	420.078363	0.2923	7
423.093385	$[C_{19}H_{20}O_{11} - H]^-$	423.093285	0.2359	10
431.098415	$[C_{21}H_{20}O_{10} - H]^-$	431.09837	0.1033	12
432.187616	$[C_{19}H_{30}O_7 + NO_3^-]^-$	432.18752	0.223	5
433.041361	$[C_{19}H_{14}O_{12} - H]^-$	433.041249	0.258	13
434.203238	$[C_{19}H_{32}O_7 + NO_3^-]^-$	434.20317	0.1563	4
435.129762	$[C_{21}H_{24}O_{10} - H]^-$	435.129671	0.2097	10
439.12463	$[C_{20}H_{24}O_{11} - H]^-$	439.124585	0.101	9
447.056997	$[C_{20}H_{16}O_{12} - H]^-$	447.0569	0.2188	13
447.093296	$[C_{21}H_{20}O_{11} - H]^-$	447.093285	0.0256	12
448.182465	$[C_{19}H_{30}O_8 + NO_3^-]^-$	448.182434	0.069	5
449.109047	$[C_{21}H_{22}O_{11} - H]^-$	449.108935	0.2484	11
461.072646	$[C_{21}H_{18}O_{12} - H]^-$	461.07255	0.2096	13
462.125339	$[C_{19}H_{32}O_7 + NO_3^-]^-$	462.125313	0.0556	7
463.051857	$[C_{20}H_{16}O_{13} - H]^-$	463.051814	0.0923	13
463.088248	$[C_{21}H_{20}O_{12} - H]^-$	463.0882	0.1037	12
463.124679	$[C_{22}H_{24}O_{11} - H]^-$	463.124585	0.2033	11
465.103957	$[C_{21}H_{22}O_{12} - H]^-$	465.10385	0.2303	11
467.083208	$[C_{20}H_{20}O_{13} - H]^-$	467.083114	0.1996	10
476.041488	$[C_{27}H_{10}O_5 + NO_3^-]^-$	476.04119	0.6272	23
477.103882	$[C_{22}H_{22}O_{12} - H]^-$	477.10385	0.0673	12
479.08321	$[C_{21}H_{20}O_{13} - H]^-$	479.083114	0.1997	12
481.135243	$[C_{22}H_{26}O_{12} - H]^-$	481.13515	0.1939	10
483.078148	$[C_{20}H_{20}O_{14} - H]^-$	483.078029	0.2475	11
486.088974	$[C_{19}H_{20}O_{11} + NO_3^-]^-$	486.088928	0.0944	10
494.094106	$[C_{21}H_{20}O_{13} + NO_3^-]^-$	494.094013	0.1871	11
495.078074	$[C_{21}H_{20}O_{14} - H]^-$	495.078029	0.0913	12
498.125422	$[C_{21}H_{24}O_{10} + NO_3^-]^-$	498.125313	0.2177	10
510.088847	$[C_{21}H_{20}O_{11} + NO_3^-]^-$	510.088928	−0.1586	12
511.093467	$[C_{18}H_{24}O_{17} - H]^-$	511.094073	−1.1847	7
512.10463	$[C_{21}H_{22}O_{11} + NO_3^-]^-$	512.104578	0.1007	11
516.099568	$[C_{20}H_{22}O_{12} + NO_3^-]^-$	516.099493	0.1464	18
526.083904	$[C_{21}H_{20}O_{12} + NO_3^-]^-$	526.083843	0.1162	12
528.09952	$[C_{21}H_{22}O_{12} + NO_3^-]^-$	528.099493	0.0525	11
535.130604	$[C_{21}H_{28}O_{16} - H]^-$	535.130458	0.2717	8
542.078849	$[C_{21}H_{20}O_{13} + NO_3^-]^-$	542.078757	0.1699	12
546.073677	$[C_{20}H_{20}O_{14} + NO_3^-]^-$	546.073672	0.0096	11
558.073831	$[C_{21}H_{20}O_{14} + NO_3^-]^-$	558.073672	0.2852	12
577.135292	$[C_{30}H_{26}O_{12} - H]^-$	577.13515	0.247	18
583.109439	$[C_{28}H_{24}O_{14} - H]^-$	583.109329	0.1883	17
599.104257	$[C_{28}H_{24}O_{15} - H]^-$	599.104244	0.0227	17
617.115001	$[C_{28}H_{26}O_{16} - H]^-$	617.114808	0.3124	16
621.14639	$[C_{28}H_{30}O_{16} - H]^-$	621.146108	0.4527	14
632.089409	$[C_{27}H_{22}O_{14} + NO_3^-]^-$	632.089322	0.1385	17
633.073511	$[C_{27}H_{22}O_{18} - H]^-$	633.073337	0.2747	17
635.089114	$[C_{27}H_{24}O_{18} - H]^-$	635.088988	0.1996	16
639.15681	$[C_{28}H_{32}O_{17} - H]^-$	639.156673	0.2141	13
640.130927	$[C_{30}H_{26}O_{12} + NO_3^-]^-$	640.130793	0.2095	18
646.10501	$[C_{28}H_{24}O_{14} + NO_3^-]^-$	646.104972	0.0597	16
662.099927	$[C_{28}H_{24}O_{15} + NO_3^-]^-$	662.099887	0.0613	17

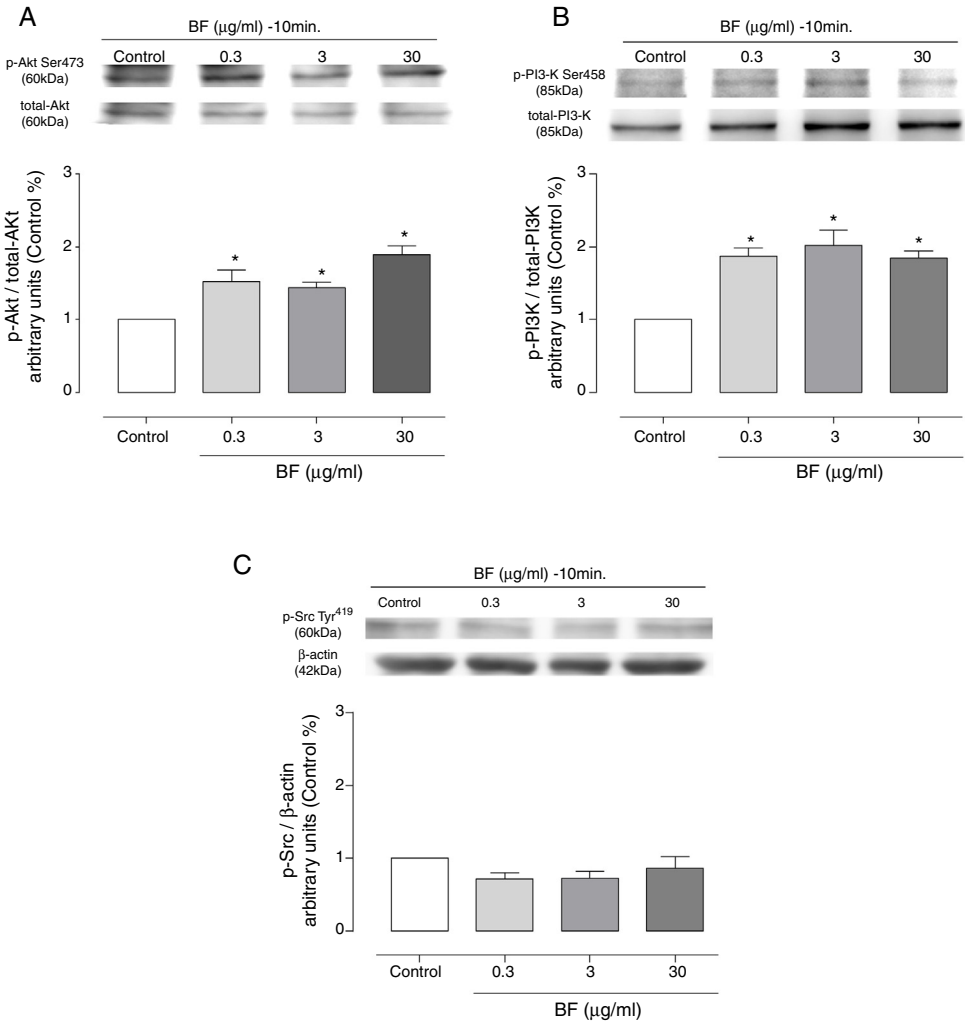


Fig. 3. The effects of the butanolic fraction (BF) of pequi leaves (*Caryocar brasiliense*) BF (0.3, 3, and 30 μg/ml) on Akt (A), PI3K (B), and Src (C) phosphorylation were determined in endothelium-intact rat thoracic aorta with intact endothelium. Data are expressed as the means ± SEM of 4–5 different immunoblots (each one corresponding to different animals), and bar graphs are representative of the relative expression of p-Akt and p-PI3K, normalized by total-AKT or total-PI3K, respectively, and p-Src protein normalized by β-actin. $^{*}p < 0.05$ versus control (ANOVA and post hoc test Tukey–Kramer).

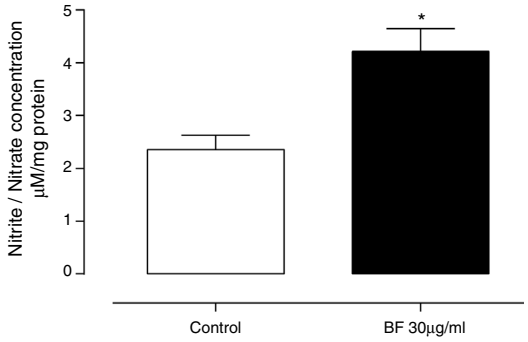
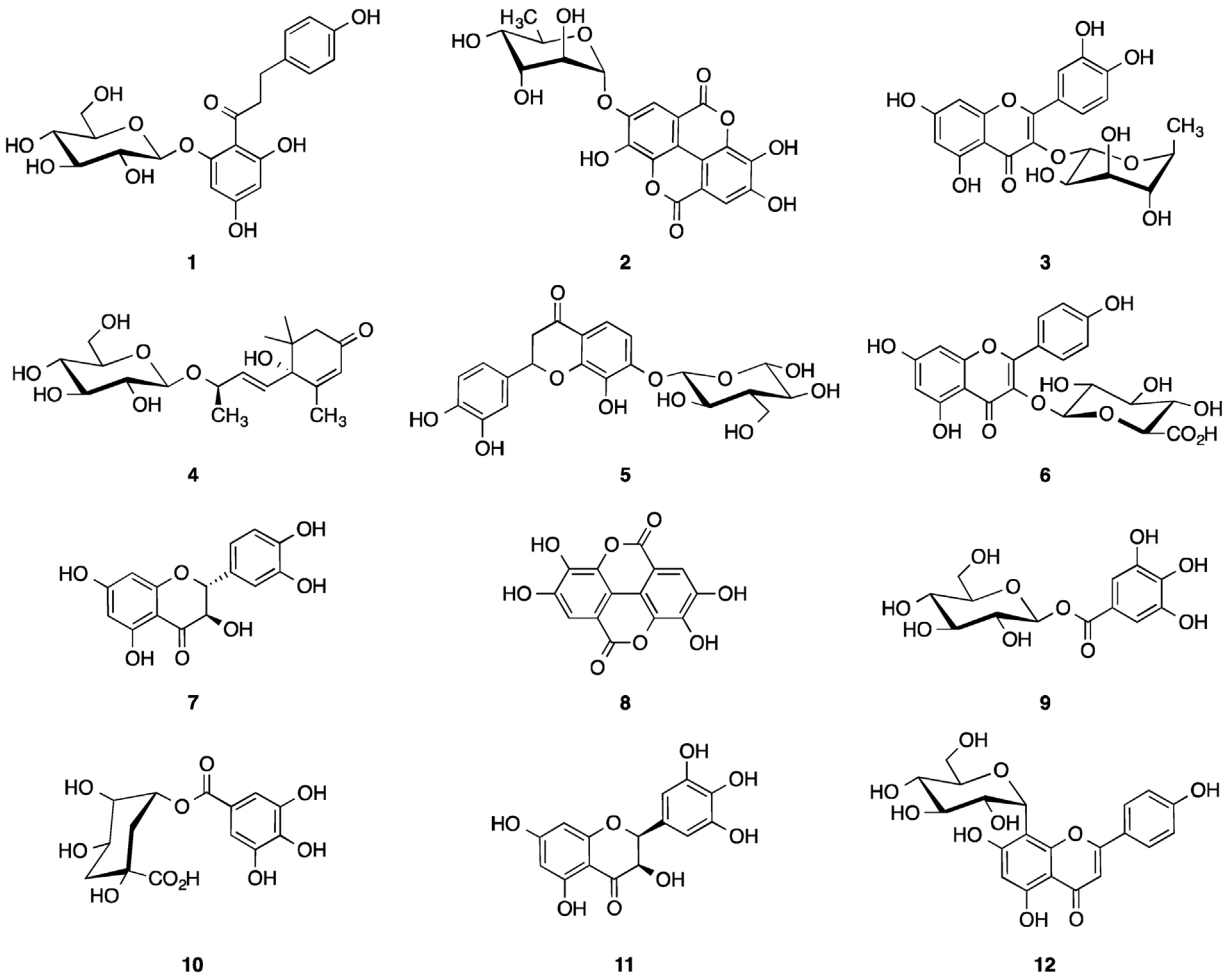


Fig. 4. The butanolic fraction (BF) of pequi leaves (*Caryocar brasiliense*). Effects of the BF increase nitric oxide (NO) production. Nitric oxide was determined by the measurement of NO metabolites nitrate and nitrite in endothelium-intact aortic rings incubated with the BF (30 μg/ml) or a vehicle (control). Bars represent the means ± SEM of 5–6 experiments (each one corresponding to different animals). $^{*}p < 0.05$ versus control (unpaired *t* test).

The information provided by the ultra-high accuracy formulas delivered by the ESI FT ICR MS measurements (error <1 ppm), along with the use of the chemspider data base (www.chemspider.com), allowed for the structural assignment and molecular formula of the BF compounds. The structure assignments are compatible with the molecular formula assigned by the ESI FT-ICR MS spectrum. Considering that the all-molecular formula shown in Table 1 has a high DBE value, it is possible to suggest that most of the compounds of the BF are phenolic compounds and their derivatives. Several of the identified compounds have been identified such as phloridzin (**1**), eschweilenol C (**2**), flavanomarein (**5**), catechin (**7**), β-glucogallin (**9**), teogallin (**10**), vitexin (**12**) and compounds with vasorelaxant activity, such as quercitrin (**3**) (Calderone et al., 2004), roseoside (**4**) (Lee et al., 2002), kaempferol (**6**) (Xu et al., 2015), ellagic acid (**8**) (Yilmaz and Usta, 2013), and epigallocatechin (**11**) (Kim et al., 2007).



Polyphenols are important constituents of plant origin, and they are present in the fruits, vegetables, and beverages in the human diet (Landete, 2012). Red wine, originating from grapes, contains large amounts of phenolic components (especially kaempferol) correlated with vasodilatory effects, which confer on them unique features in the prevention of cardiovascular disease (Padilla et al., 2005). Similar to wines, the cardioprotective benefits of tea are attributed to polyphenol compounds, as described for epigallocatechin, a green tea polyphenol and also identified in the BF that mediates NO-dependent vasorelaxation (Kim et al., 2007).

Considering that the relationship between vasodilation capacity and phenolic content has been extensively described (Andriambeloson et al., 1997, 1998; Padilla et al., 2005; Yung et al., 2008; Landete, 2012) and that the compounds identified in the BF are predominantly polyphenols, we can suggest that the vasorelaxant effect of the BF is associated with phenolic compounds. However, further pharmacological studies will be carried out to identify the active polyphenolic compound(s) of the BF.

Conclusion

The findings here showed that the calmodulin and phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathways are

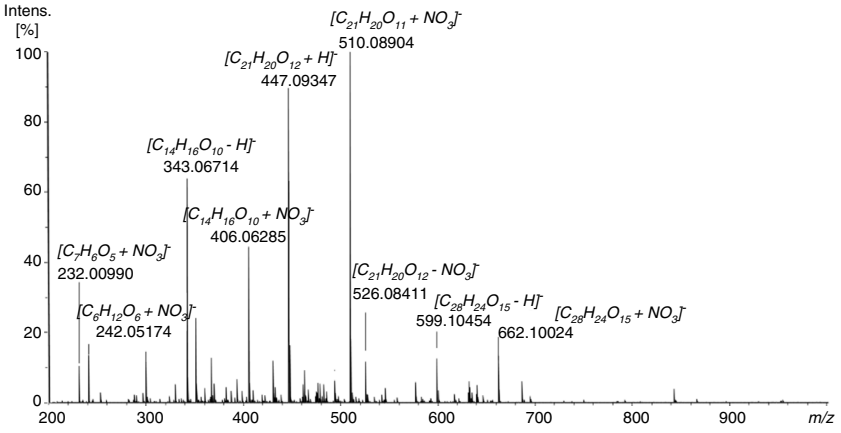


Fig. 5. Electrospray Ionization Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (ESI FT-ICR MS) for the butanolic fraction (BF) of *Caryocar brasiliense* leaves.

involved in the BF-induced endothelial NO synthase (eNOS) activation and are promoted by polyphenol compounds present in the *C. brasiliense* leaves.

Authors contributions

LMO, TSO, RMC, JLRM and CSF were responsible for the acquisition, analysis and interpretation of the data. ESG, EAC, RCATP and GBV were responsible for the analysis and interpretation of data, and contributed with new reagents and analytical tools. FPF and PCG were responsible for the concept and design of the manuscript, and for the preparation of the manuscript. All authors read and approved the final version of the manuscript.

Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

The experimental protocols were performed in accordance with the Ethical Principles in Animal Experimentation adopted by the Brazilian College of Animal Experimentation (COBEA) and were approved by the local Ethics in Research Committee (Protocol CEP/UFG 22/2011). The authors declare that no experiments were performed on humans.

Confidentiality of data. The authors declare that they have followed the protocols of their work center on the publication of patient data.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Conflicts of interest

The authors declare no conflicts of interest.

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References

Abraham, S.T., Benscoter, H.A., Schworer, C.M., Singer, H.A., 1997. A role for Ca^{2+}/calmodulin-dependent protein kinase II in the mitogen-activated protein kinase signaling cascade of cultured rat aortic vascular smooth muscle cells. Circ. Res. 81, 575–584.

Almeida, S.P., Silva, J.A., 1994. Piqui e buriti – importância alimentar para a população dos Cerrados. Empresa Brasileira de Pesquisa Agropecuária do Brasil (EMBRAPA – CPAC). Brasil, 10–30.

Andriambeloson, E., Kleschyov, A.L., Muller, B., Beretz, A., Stoclet, J.C., Andriantsitohaina, R., 1997. Nitric oxide production and endothelium-dependent vasorelaxation induced by wine polyphenols in rat aorta. Br. J. Pharmacol. 120, 1053–1058.

Andriambeloson, E., Magnier, C., Haan-Archipoff, G., Lobstein, A., Anton, R., Beretz, A., Stoclet, J.C., Andriantsitohaina, R., 1998. Natural dietary polyphenolic compounds cause endothelium-dependent vasorelaxation in rat thoracic aorta. J. Nutr. 128, 2324–2333.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Brandão, M., Laca-Buendía, J.P., Macedo, J.F., 2002. Árvores nativas e exóticas do Estado de Minas Gerais. Belo Horizonte: Empresa Brasileira de Pecuária de Minas Gerais, 528.

Calderone, V., Chericoni, S., Martinelli, C., Testai, L., Nardi, A., Morelli, I., Breschi, M.C., Martinotti, E., 2004. Vasorelaxing effects of flavonoids: investigation on the possible involvement of potassium channels. Naunyn Schmiedebergs Arch. Pharmacol. 370, 290–298.

de Oliveira, L.M., Rodrigues, A.L., Silva, E.F., Cerqueira, L.B., Castro, C.H., Pedrino, G.R., de Carvalho, M.H.C., Pontarolo, R., Costa, E.A., Campos, F.R., Filgueira, F.P., Ghedini, P.C., 2012. Endothelium-dependent vasorelaxant effect of butanolic fraction from *Caryocar brasiliense* Camb. leaves in rat thoracic aorta. Evid. Based Complement. Alternat. Med., http://dx.doi.org/10.1155/2012/934142.

de Oliveira, L.M., de Oliveira, T.S., da Costa, R.M., de Souza Gil, E., Costa, E.A., Passaglia, R. de C., Filgueira, F.P., Ghedini, P.C., 2016. The vasorelaxant effect of gallic acid involves endothelium-dependent and -independent mechanisms. Vascul. Pharmacol. 81, 69–74.

de Paula-Junior, W., Rocha, F.H., Donatti, L., Fadel-Picheth, C.M.T., Weffort-Santos, A.M., 2006. Leishmanicidal, antibacterial, and antioxidant activities of *Caryocar brasiliense* Cambess leaves hydroethanolic extract. Rev. Bras. Farmacogn. 16, 625–630.

Dimmeler, S., Fleming, I., Fisslthaler, B., Hermann, C., Busse, R., Zeiher, A.M., 1999. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. Nature 399, 601–605.

Ferreira, F.P.S., Morais, S.R., Bara, M.T.F., Conceição, E.C., Paula, J.R., Carvalho, T.C., Vaz, B.G., Costa, H.B., Romao, W., Rezende, M.H., 2014. *Eugenia calycina* Cambess extracts and their fractions: their antimicrobial activity and the identification of major polar compounds using electrospray ionization FT-ICR mass spectrometry. J. Pharm. Biomed. Anal. 99, 89–96.

Fleming, I., Busse, R., 1999. Signal transduction of eNOS activation. Cardiovasc. Res. 43, 532–541.

Fleming, I., Busse, R., 2003. Molecular mechanisms involved in the regulation of the endothelial nitric oxide synthase. Am. J. Physiol. Regul. Integr. Comp. Physiol. 284, 1–12.

Forstermann, U., Munzel, T., 2006. Endothelial nitric oxide synthase in vascular disease: from marvel to menace. Circulation 113, 1708–1714.

Fulton, D., Gratton, J.P., McCabe, T.J., Fontana, J., Fujio, Y., Walsh, K., Franke, T.F., Papapetropoulos, A., Sessa, W.C., 1999. Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. Nature 399, 597–601.

Khouri, J., Resck, I.S., Poças-Fonseca, M., Sousa, T.M.M., Pereira, L.O., Oliveira, A.B.B., Grisolia, C.K., 2007. Anticlastogenic potential and antioxidant effects of an aqueous extract of pulp from the pequi tree (*Caryocar brasiliense* Camb). Genet. Mol. Biol. 30, 442–448.

Kim, J.A., Formoso, G., Li, Y., Potenza, M.A., Marasciulo, F.L., Montagnani, M., Quon, M.J., 2007. Epigallocatechin gallate, a green tea polyphenol, mediates NO-dependent vasodilation using signaling pathways in vascular endothelium requiring reactive oxygen species and Fyn. J. Biol. Chem. 282, 13736–13745.

Landete, M., 2012. Updated knowledge about polyphenols: functions, bioavailability, metabolism, and health. Crit. Rev. Food Sci. Nutr. 52, 936–948.

Lee, T.H., Wang, G.J., Lee, C.K., Kuo, Y.H., Chou, C.H., 2002. Inhibitory effects of glycosides from the leaves of *Melaleuca quinquenervia* on vascular contraction of rats. Planta Med. 68, 492–496.

Li, P.G., Sun, L., Han, X., Ling, S., Gan, W.T., Xu, J.W., 2012. Quercetin induces rapid eNOS phosphorylation and vasodilation by an Akt-independent and PKA-dependent mechanism. Pharmacology 89, 220–228.

Lorenzi, H., 1998. Árvores Brasileiras: manual de identificação e cultivo de plantas arbóreas nativas do Brasil, 2 ed., Brazil.

Magalhães, H.G., Monteiro, H., Lagrota, M.H., Wigg, M.D., Guimarães, L.A.S., Loja, M.A.S.O., Araújo, R.R., 1988. Estudo estrutural do pequizeiro *Caryocar brasiliense* Camb. Caryocaraceae, sob o aspecto farmacoquímico e botânico. Rev. Bras. Farm 69, 31–41.

Miranda-Vilela, A.L., Resck, I.S., Grisolia, C.K., 2008. Antigenotoxic activity and antioxidant properties of organic and aqueous extracts of pequi fruit (*Caryocar brasiliense* Camb.) pulp. Genet. Mol. Biol. 31, 956–963.

Mors, W.B., Rizzini, C.T., Pereira, N.A., 2000. Medicinal Plants of Brasil. Reference Publications Inc., Algonac, MI, pp. 501.

Mount, P.F., Kemp, B.E., Power, D.A., 2007. Regulation of endothelial and myocardial NO synthesis by multi-site eNOS phosphorylation. J. Mol. Cell Cardiol. 42, 271–279.

Oliveira, M.M., Gilbert, B., Mors, W., 1968. Triterpenes in *Caryocar brasiliense*. An. Acad. Bras. Cien. 40, 451.

Padilla, E., Ruiz, E., Redondo, S., Gordillo-Moscoso, A., Slowing, K., Tejerina, T., 2005. Relationship between vasodilation capacity and phenolic content of Spanish wines. Eur. J. Pharmacol. 4, 84–91.

Roesler, R., Catharino, R.R., Malta, L.G., Eberlin, M.N., Pastore, G., 2008. Antioxidant activity of *Caryocar brasiliense* (pequi) and characterization of components by electrospray ionization mass spectrometry. Food Chem. 110, 711–717.

Sant'Anna, L.S., Merlugo, L., Ehle, C.S., Limberger, J., Fernandes, M.B., Santos, M.C., Mendez, A.S.L., Paula, F.R., Moreira, C.M., 2017. Chemical composition and hypotensive effect of *Campomanesia xanthocarpa*. Evid. Based Complement Alternat. Med., http://dx.doi.org/10.1155/2017/1591762.

Shaul, P.W., 2002. Regulation of endothelial nitric oxide synthase: location, location, location. Annu. Rev. Physiol. 64, 749–774.

Stuehr, D.J., 1997. Structure–function aspects in the nitric oxide synthases. Annu. Rev. Pharmacol. Toxicol. 37, 339–359.

Xu, Y.C., Leung, S.W., Leung, G.P., Man, R.Y., 2015. Kaempferol enhances endothelium-dependent relaxation in the porcine coronary artery through activation of large-conductance Ca(2+)-activated K(+) channels. Br. J. Pharmacol. 172, 3003–3014.

Yilmaz, B., Usta, C., 2013. Ellagic acid-induced endothelium-dependent and endothelium-independent vasorelaxation in rat thoracic aortic rings and the underlying mechanism. Phytother. Res. 27, 285–289.

Yung, L.M., Leung, F.P., Wong, W.T., Tian, X.Y., Yung, L.H., Chen, Z.Y., Yao, X.Q., Huang, Y., 2008. Tea polyphenols benefit vascular function. Inflammopharmacology 16, 230–234.