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Endothelium-independent vasorelaxation by dichloromethanolic fraction from *Anogeissus leiocarpa* (DC) Guill. Et Perr. (Combretaceae) bark of trunk on porcine coronary artery rings: Involvement of $[Ca^{2+}]_i$ decreased and phosphodiesterases inhibition

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***Anogeissus leiocarpa* (DC) Guill. et Perr. belongs to the Combretaceae family and was previously named *A. leiocarpa* (DC) Guill. Et Perr. It has been widely used in Burkina Faso by traditional medicine for the treatment of hypertension. Previous study showed that the dichloromethanolic fraction from the barks of trunk of *A. leiocarpa* (ALF) has induced an endothelium-independent and endothelium-dependent vasodilation effect and had the capacity to inhibit *in vitro*, purified cyclic nucleotide phosphodiesterases (PDEs) activity. The aims of this study were to better underline ALF-induced endothelium-independent vasorelaxation in an organ model. The results showed that ALF significantly reduce the contractile response to U46619 in porcine coronary artery rings without endothelium that were in concentration-dependent manner. In denuded rings, pretreatment by ALF (10, 30 and 100 µg/mL) did not affect relaxation to sodium nitroprusside (SNP), suggesting that relaxation to ALF was not due to its ability to be a nitric oxide donor. Moreover, SNP-induced relaxation had not been affected in the presence of Nimodipine (PDE1 inhibitor), EHNA (PDE2 inhibitor) or DMPPO (PDE5 inhibitor). In addition, the results showed a relaxation effect to isoproterenol in endothelium-denuded artery rings pretreated with ALF (3, 10, and 30 µg/mL) which were significantly affected suggesting a possible membrane hyperpolarization leading to the vasodilation. In the presence of the PDE3 specific inhibitor Cilostamide and ALF (30 and 100 µg/mL), the vasodilation effects of isoproterenol was enhanced and comparable. Moreover, various potassium channels were not involved in ALF-induced relaxation since tetraethylammonium chloride (non-selective K^+ channels inhibitor), iberiotoxin (voltage-sensitive potassium channels inhibitor) and Glibenclamide (K_{ATP} channels inhibitor) did not notably affect the relaxation effect to ALF in rings without endothelium. Taken together, ALF-induced endothelium-independent relaxation mainly involves a sustained decrease in $[Ca^{2+}]_i$ and may be due to PDE1, 3 and 5 inhibitions localized in the vascular smooth muscle cells. While, the involvement of the ions channels have not been clearly revealed in this experiment.**

Key words: Anogeissus leiocarpa, U46619, phosphodiesterases (PDEs), calcium, porcine coronary artery, sodium nitroprusside (SNP), isoproterenol.

INTRODUCTION

The traditional medicine has always been used in Burkina Faso for the care of the population and this for several generations. In cardiovascular diseases such as stroke, headache, and hypertension, vessels dilation is fundamental because it directly influences the arteries of the circulatory system. Accordingly, many researchers have investigated the vasorelaxant effects of various herbal medicines (Gan et al., 2016; Kassahun Gebremeskel et al., 2017; Cam et al., 2018; Khan et al., 2018; Vajic et al., 2018; Sanchez-Recillas et al., 2019). It was the case of a recent study which indicated the endothelium-dependent vasodilation effect of the aqueous extract of *Anogeissus leiocarpa* (Belemnaba et al., 2018).

Anogeissus leiocarpa is commonly named African birch. It has large ecological distribution ranging from the boarders of Sahara up to the out layer humid tropical forests. In West Africa, it expands from Senegal to Cameroon, with extension to Ethiopia and East Africa. It has been grown in dry and gallery forests. The tree was up to 30 m in height but typically, between 15 and 18 m with light green foliage. The base of the trunk is wider and occasionally striped. The colour of the bark is grey and becomes blackished depending on the age. It is fibrous with thin scales. It has a finely publescent stems and alternate to sub-opposite, elliptical to oval leaves which are 2 to 8 cm length and 1.5 to 3.5 cm wide (Arbonnier, 2009).

However, there have been fewer pharmacological studies and clinical data for *A. leiocarpa* in comparison to other medicinal plants used for their pharmacological effects through their molecules contain (*Notopterygii Rhizoma* and *Radix*; *Calotropis procera*, *Kaya senegalensis*, *Moringa oleifera*).

Therefore, more pharmacological and clinical studies are needed to support the continuous use of *A. leiocarpa* in traditional medicine for the treatment of hypertension.

Nevertheless, studies on *A. leiocarpa* extracts had already reported various pharmacological activities such as antioxidant, antimicrobial, anthelmintic activity and antihypertensive effect (Mann et al., 2008; Konaté et al., 2011; Soro et al., 2013; Ouedraogo and Kiendrebeogo, 2016). Many second messengers are involved in the regulation of the cardiovascular system with specifications depending on whether it is at the heart or vascular level. At the heart level, regulation involves several systems such as the orthosympathetic and parasympathetic systems in which intracellular Ca^{2+} as the main regulator of cardiac excitation-contraction coupling. An imbalance in the intracellular calcium concentration inevitably leads to heart dysfunction (Chung et al., 2016; Mora et al., 2017; Montalvo et al., 2018). At the vascular level, regulation in the presence of endothelium involves many second messengers such as nitric oxid, prostacyclin I_2, inhibition of phosphodiesterases (PDEs), endothelium-derived hyperpolarizing factors and many others messengers. In the absence of endothelium, this relaxation notably involves the potassium channels, calcium channels, inhibition of PDEs and other vasodilation factors (Alamgeer et al., 2018; Jia et al., 2018; Sanchez-Recillas et al., 2019). Specifically for *A. leiocarpa* fraction (ALF), previous studies have shown that this extract has induced an endothelium-dependent and endothelium-independent vasorelaxant effect on pig artery by involving mostly NO/sGC/cGMP and Na^+/k^+-ATPase pathways and in some extent a possible PDEs inhibition (Belemnaba et al., 2013). However, there was no published studies on this extract demonstrating that its vasorelaxation effect implicates the inhibition of PDEs in a vascular system. Indeed, the possible implication of the PDEs inhibition in the mechanism of vasodilation of ALF has been demonstrated but through PDEs purified according to a tube model (Belemnaba et al., 2013). It was therefore necessary to show this implication on a model using vessels which is close to reality. Moreover, it was well known that other ions channels were involved in vascular stone regulation especially in endothelium independent arteries. It was the case of large-conductance calcium-activated potassium channels (BK_{Ca}), K_{ATP} channels and K^+ channels (Gan et al., 2016; Greenberg et al., 2016; Li et al., 2018).

For this purpose, we have used pig isolated arteries rings to assess the implication of PDEs inhibitions in the ALF endothelium independent vasodilation effects by using various pharmacologicals agents [bradykinin, sodium nitroprusside (SNP, a NO donor), isoproterenol (Adenylate cylase activator)] and specifc inhibitors [Nimodipine (PDE1 inhibitor), EHNA (PDE2 inhibitor), Cilostamide (PDE3 inhibitor), DMPPO (PDE5 inhibitor), tetraethylammonium chloride (non-selective K^+ channels inhibitor), iberiotoxin (voltage-sensitive potassium channels inhibitor) and Glibenclamide (K_{ATP} channels inhibitor)].

MATERIALS AND METHODS

Plant and extraction

The plant materials consist of the barks of the trunk of *A. leiocarpa*. The collection method of *A. leiocarpa* has been previously described (Belemnaba et al., 2013).

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Briefly, the barks of trunk of *A. leiocarpa* were collected in May 2006 in the East of Ouagadougou (zone of savana). A voucher specimen (Number 1544) was deposited at the Department of Forest Production of the National Centre for Scientific and Technological Research (CNRST) after identification by a botanist of this centre. Plant material freshly collected was dried in a greenhouse with air circulation. It was powdered in a pulverizer mil and a decoction was made (120 g in 2100 mL distilled water). After the decoction, ALF extract was obtained by an exhaustion into a methylene chloride solution, then concentrated into a rotavapor system and finally dried in an oven at 35°C.

Drugs and chemicals

Bradykinin, isoproterenol (Isop), indomethacin, sodium nitroprusside (SNP), glibenclamide (GLIB), iberiotoxin (IBTX), tetraetylammonium chloride (TEA), bradykinin and the cyclic nucleotides PDE inhibitors (EHNA, Cilostamide) were from Sigma-Aldrich (Saint Quentin Fallavier, France). Nimodipine and DMPPO were given by Bayer (Berlin). U46619 (9,11-dideoxy-11α,9α-epoxymethanoprostaglandin F2α) was from Cayman Chemical (Ann Arbor, MI, United States of America).

Vascular reactivity studies

The vascular reactivity of ALF was assessed as indicated previously (Belemnaba et al., 2018). Pig hearts were collected from the local slaughterhouse (Copvial, Holtzheim) and left circumflex coronary arteries were excised and then cleaned to remove all connectives tissues. Porcine coronary arteries were cut into rings of 3 to 4 mm and suspended in organ bath contained appropriated krebs bicarbonate solution (Composition in mM: NaCl 119, $CaCl_2$ 1.25, $NaHCO_3$ 25, $MgSO_4$ 1.18, KH_2PO_4 1.18, KCl 4.7, D-glucose 11, pH 7.4, 37°C) and oxygened with carbogen gas (mixture of oxygen 95% and carbon dioxide 5%). Rings were put into an initial isometric tension of 5 g for 1 h.

The presence of endothelium was confirmed pharmacologically by testing the response to bradykinin at 3×10^{-7} M under pre-contraction conditions with U46619. A relaxation about 90% of the contracted rings by bradykinin (3×10^{-7} M) was considered with endothelium. When required, the endothelium was removed by gently rubbing the intimal space with a stainless steel rod with a diameter equivalent to that of the arterial lumen. A relaxation about 10% of the maximal contracted rings by bradykinin (3×10^{-7} M) was considered without endothelium. For the assessment of ALF effect on rings, rings were contracted with U46619 to about 80% of the maximal contraction before the construction of the concentration-response curve to ALF. In some experiments, rings were pre-incubated with specific inhibitors or ALF (3, 10, 30 or 100 µg/mL) before contraction to U46619 and the subsequent construction of a concentration-response curve to an antagonist. Indeed, to determine the effect of the extract on U46619-induced vasoconstriction, rings were first incubated with ALF (3, 10, 30, 100 and 300 µg/mL) for 5 min before a cumulative of U46619. In other experiments, rings were pre-incubated with different concentrations of ALF (3, 10, 30, 100 and 300 µg/mL) for 5 min followed by U46619 to the maximum contraction and subsequent cumulative with SNP or isoproterenol. Other rings were first pre-incubated with PDEs inhibitors (Nimodipine, EHNA, Cilostamide, DMPPO) or with TEA, Glibenclamide or Iberiotoxin for 30 min followed by U46619 contraction and cumulative with ALF (3, 10, 30, 100 and 300 µg/mL).

Statistical analysis

All results were expressed as the mean ± standard error of mean (SEM) and *n* represents the number of rings from different pigs used in the experiments. The relaxation response was expressed as percentage of decreases in tension from the contracting level induced by U46619. The concentrations of substances induced 50% of maximal relaxation (EC_{50}) and their maximal relaxation (Emax) was determined with GraphPad Prism 5.00.288. Two way or one way ANOVAs (with post hoc Bonferroni's test) determined significant differences, if any, between concentration-relaxation curves and EC_{50}, respectively in different treatment groups. $P < 0.05$ was considered as the significant threshold.

RESULTS

Effect of ALF (3-100 µg/mL) on U46619-induced contraction in rings with endothelium

The thromoxane A2 mimetic U46619 (10^{-10} M to 3×10^{-7} M) has induced a concentration-dependent contraction of pig coronary rings (Figure 1). The magnitude of U46619-induced isometric tension development on rings without endothelium was in a concentration-dependent manner. The maximum contraction-response (Emax) to U46619 in rings pre-incubated with ALF 3, 10, 30 and 100 µg/mL was of 21.57±1.88, 20.73±1.81, 17.04±1.56 and 13.11±0.51 g, respectively (Figure 1B) while the control was 20.34±1.69 g. The pre-incubation by ALF 30 and 100 µg/mL causes a significant reduction in the contraction-induced by U46619 as compared to the control. Moreover, we noted a slight but not significant shift to the left and to the right contraction-curve when rings were preincubated with ALF 3 and 10 µg/mL, respectively compared to the control.

Effects of ALF (3-100 µg/mL) on SNP-induced vasodilation in porcine arteries rings

The results showed that the endothelium-independent vasodilator sodium nitroprusside (SNP, 10^{-11} to 10^{-5} M) was dose-dependently relaxed U46619-contracted pig coronary arteries rings without endothelium. In pre-incubated denuded artery rings with ALF (3, 10, 30 or 100 µg/mL), the vasodilator effect of SNP (a NO donnor) was significantly improved in a concentration-dependent manner compared to the control (Figure 2). The half-maximal effective concentration (EC_{50}) values were of 0.171±0.076 µM for the control, 0.054±0.019, 0.050±0.034, 0.043±0.017 and 0.005±0.002 µM in the presence of ALF 3, 10, 30 and 100 µg/mL, respectively. No significant difference was notified in the Emax values that were 99.51±1.72, 100.27±0.37, 101.62±1.78, 100.2±0.35, and 100±0, respectively for the control, ALF 3, 10, 30 and 100 µg/mL.

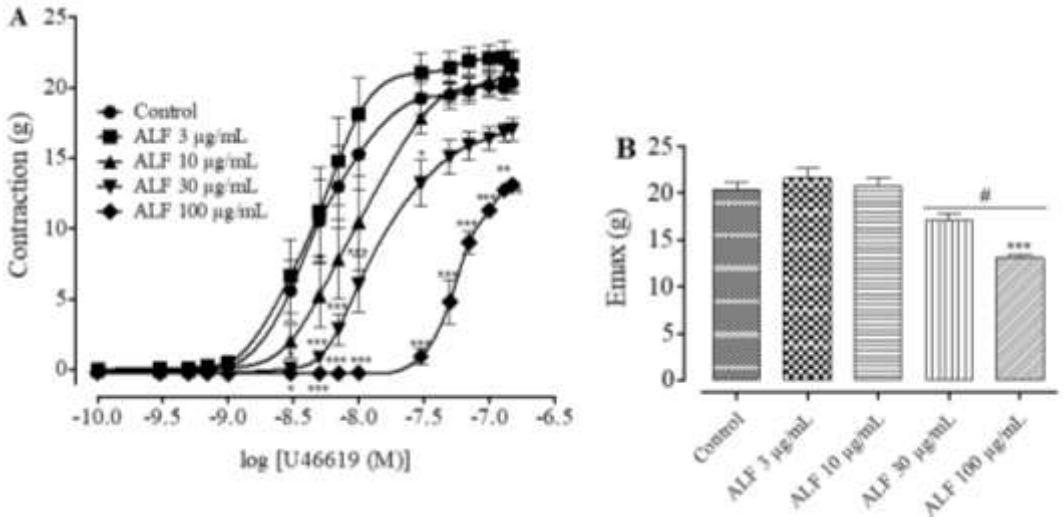

Figure 1. Effect of ALF on U46619-induced contraction : (A) Denuded porcine coronary artery rings were exposed to ALF (3, 10, 30, 10 µg/ml) 5 min before the addition of increasing concentration of U46619 ; (B) The respective maximal contraction obtained. Experiments were performed in the presence of indomethacin (n=3 to 5 different experiments; *P<0.05 vs. Control).

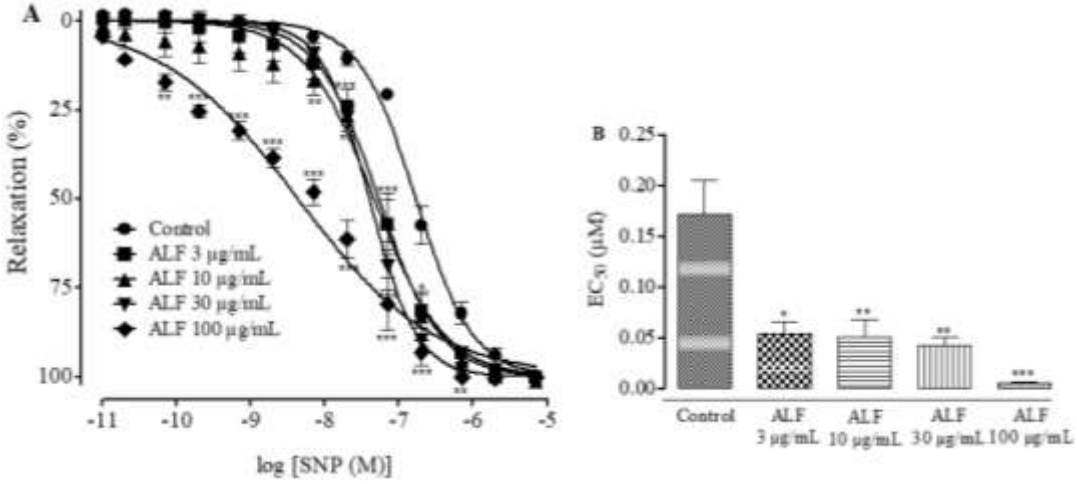

Figure 2. (A) Effect of ALF (3, 10, 30, and 100 µg/ml) on the relaxation-induced by sodium nitroprusside (SNP) in denuded porcine artery rings pre-contracted with U46619. (B) The histogram of the representative EC50 values.Experiments were performed in the presence of indomethacin (10 µM) to avoid endothelium derivative hyperpolarization factor (n=6 to 8 different experiments, *P<0.05 vs. Control).

Effects of Nimodipine (1 µM), EHNA (10 µM), and DMPPO (0.1 µM) on SNP-induced vasodilatation in porcine arteries rings

The concentration-relaxation curves to SNP in denuded rings contracted with U46619 were significantly shifted to the left when pre-incubated with Nimodipine (PDE1 specific inhibitor),EHNA (PDE2 specific inhibitor)or DMPPO(PDE5 specific inhibitor) compared to the control (Figure 3).

The EC_{50} values of SNP-induced endothelium-independent vasodilation on rings were of 0.063±0.040, 0.059±0.015 and 0.022±0.007 µM, respectively in the presence of Nimodipine (1 µM), EHNA (10 µM), and DMPPO (0.1 µM). When comparing the EC_{50} values, only the presence of DMPPO showed a significant difference compared to the control (EC_{50} = 0.171±0.076 µM) but their Emax was not affected.

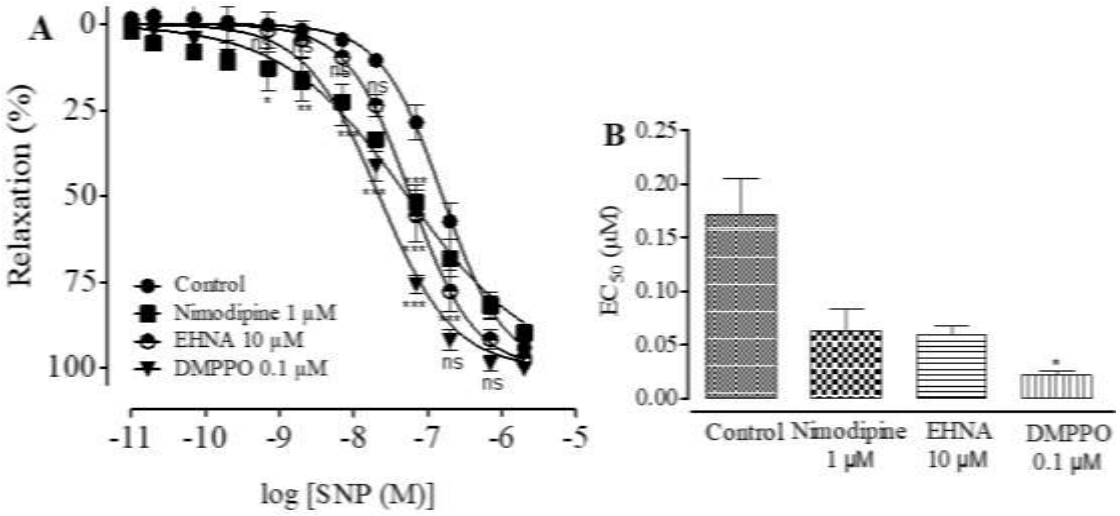

Figure 3. Characterization of SNP-induced relaxation in porcine coronary artery ring. (A) Rings without endothelium were exposed to EHNA (PDE2 specific inhibitor), Nimodipine (PDE1 specific inhibitor), and DMPPO (PD5 specific inhibitor) 30 min each, before a cumulative concentration of SNP (10^{-11} to 10^{-5} M). (B) The representative EC50 for the control and in the presence of Nimodipine (1 µM), EHNA (10 µM) and DMPPO (0.1 µM). Experiments were performed in the presence of indomethacin (10 µM) (n=4 to 5 different experiments, *P<0.05 vs. Control).

Effects of ALF (3-100 µg/mL) on isoproterenol-induced vasorelaxation in porcine arteries rings

Isoproterenol caused concentration-dependent relaxation of U46619 pre-contracted in pig coronary artery without endothelium (EC_{50} = 0.148±0.027 µM). Pre-incubation with ALF (3, 10, 30 and 100 µg/mL) has been potentiated significantly in the relaxation effect of isoproterenol in a concetration-dependent manner compared to the control. The EC_{50} was of 0.08±0.03, 0.04±0.009, 0.02±0.01 and 0.01±0.002 µM in the presence of ALF 3, 10, 30 and 100 µg/mL, respectively. However, the Emax values were not significantly modified compared to the control (Figure 4).

Effects of ALF (3 µg/mL), nimodipine (10 µM) and DMPPO (0.1 µM) on isoproterenol-induced vasodilatation in porcine arteries rings

The results shown in Figure 5 indicated that the vasodilation effect to isoproterenol was not notably improved when rings were preincubated either with nimodipine (PDE1 specific inhibitor) or with DMPPO (PDE5 specific inhibitor). The shape of their relaxation curves was similar to that following the pre-incubation with ALF 3 µg/mL. The EC_{50} values of isoproterenol were of 0.148±0.027 µM for the control and of 0.076±0.030, 0.123 ±0.091 and 0.112±0.076 µM in the presence of ALF 3 µg/mL, nimodipine and DMPPO, respectively but without significant difference to the control.

Effects of ALF (30 and 100 µg/mL) and cilostamide (10 µM) on isoproterenol-induced vasodilation in porcine arteries rings

The results showed that the vasodilation effect to isoproterenol was higly and significantly improved when rings were pre-incubated with cilostamide (a PDE3 specific inhibitor, 10 µM; Figure 6). This effect was relatively similar to those obtained in the presence of ALF30 and 100 µg/mL. The EC_{50} of isoproterenol was of 0.030±0.007, 0.023±0.007 and 0.014±0.002 µM in the presence of cilostamide, ALF 30 and 100 µg/mL, respectively. Thus, the rank orders for relaxant effect of isoproterenol when pre-incubated with ALF and cilostamide were ALF 100 µg/mL > ALF 30 µg/mL > Cilostamide.

Effects of TEA (1 µM), IBTX (10 µM) and GLIB (0.44 µM) on ALF-induced vasodilation in porcine arteries rings

In endothelium denuded artery rings, results showed that the non-selective blockade of potassium channels with TEA was not affected. ALF (0.1 to 30 µg/mL)-induced vasodilation effect that was superposable to those of the control (Figure 7). When pre-incubated with IBTX or Glibenclamide, the relaxation of ALF in rings were slighly shifted but not significantly different to the control. The Emax values were 97.41±6.84% for the control and 100%

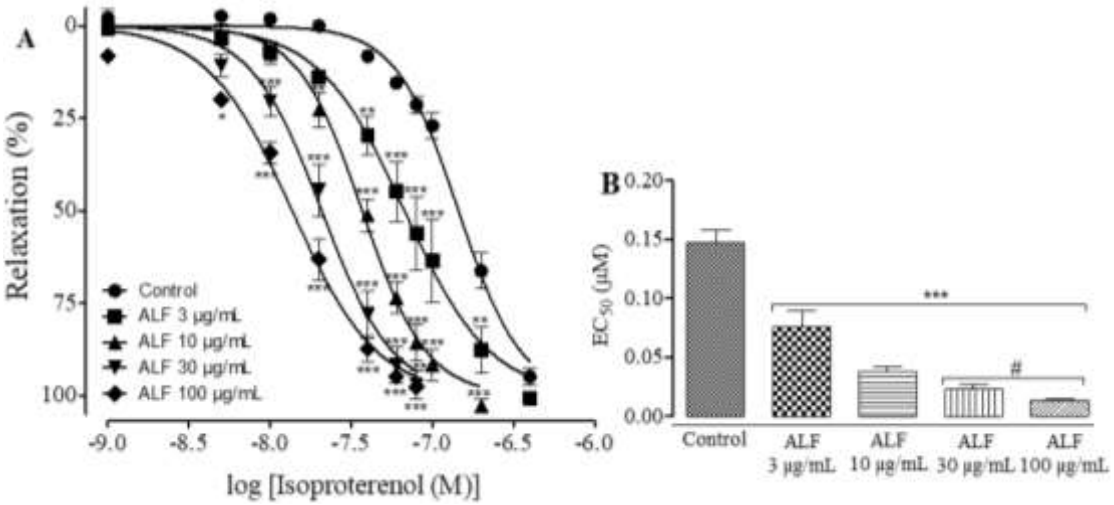

Figure 4. (A) Characterization of isoproterenol-induced relaxation in porcine coronary artery ring: endothelium-denuded rings were exposed to ALF (3, 10, and 30 µg/ml) 5 min before the addition of increasing concentration of isoproterenol (10^{-9} to 4 $\times 10^{-7}$ µM). (B) Histogram representing the relative EC50. Experiments were performed in the presence of indomethacin (n=3 to 8 different experiments, *P<0.05 vs. Control; *P<0.05 vs. ALF 3 µg/ml).

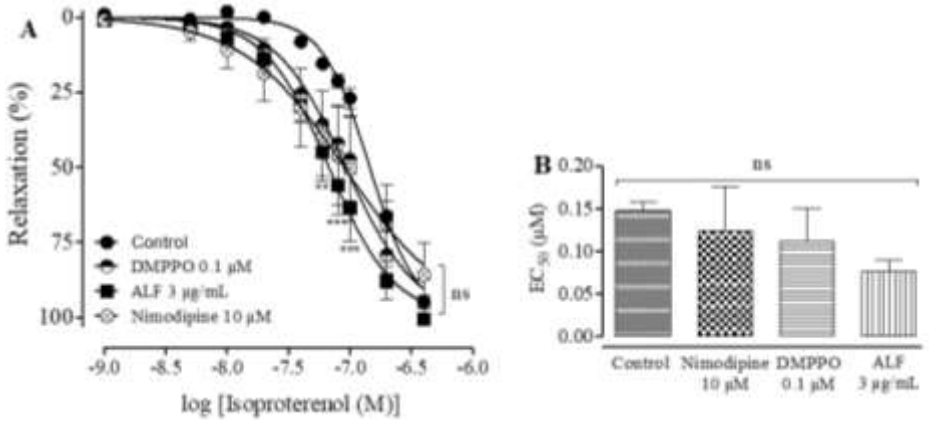

Figure 5. Effect of Nimodipine (PDE1 inhibitor, 30 min) and DMPPO (PDE5 specific inhibitor) on isoproterenol-induced relaxation in coronary artery rings without endothelium. For comparison, the effect of ALF (3 µg/ml) is induced in the graphs. (B) Histogram represent the relative EC50 for the control and in the presence of Nimodipine, DMPPO and ALF. All experiments were performed in the presence of indomethacin (10 µM) (n=4 to 8 different experiments, *P<0.05 vs. Control).

in the presence of the tested inhibitors.

DISCUSSION

In previous study, it was demonstrated that ALF has been induced as an endothelium-dependent and endothelium-independent vasodilation effect in porcine coronary arteries rings that was mediated via NO/sGC/cGMP and cAMP pathways, potassium (K^+) and partly Na^+/K^+-ATPase channels opening (Belemnaba et al., 2013). Moreover, these findings indicated that ALF was a powerful inhibitor of PDE1, 2, 4 and PDE5 with a less activity on PDE 3. While these tests were conducted on tubes assay and on purified PDEs.

In the present study, experiments were focus on the endothelium-independent vasodilaion effect of ALF in order to better characterized its underline mechanism of

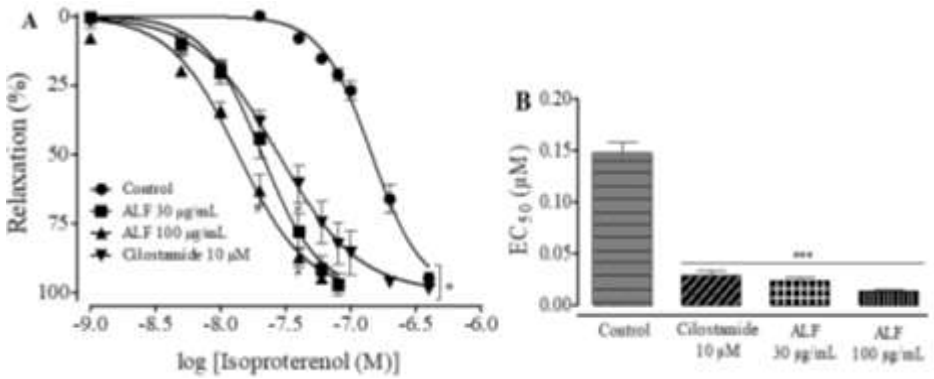

Figure 6. (A) Effect of Cilostamide (PDE3 inhibitor, for 30 min) on isoproternol-induced relaxation in denuded coronary artery rings. For comparison, the effect of ALF (3 and 100 µg/ml) were included in the graph. (B) Histogram represent the relative EC50. All experiments were performed in the presence of indomethacin (10 µM); n=4 to 8 different experiments (*P<0.05 vs. Control).

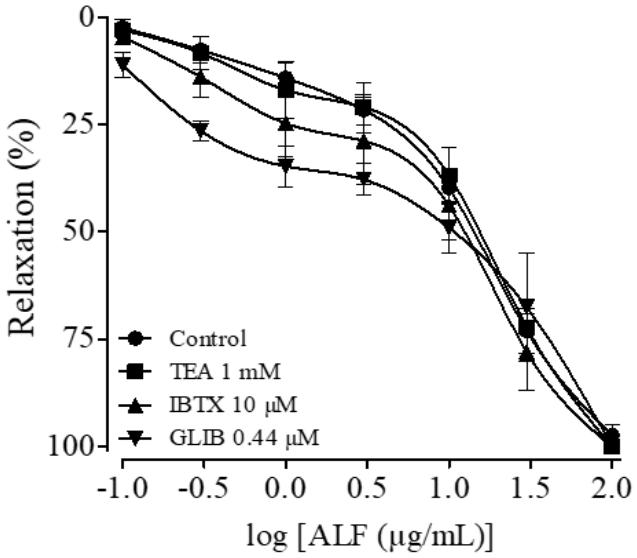

Figure 7. Cumulative-response of ALF in porcine coronary artery rings without endothelium. Rings were pre-incubated with TEA (non-selective inhibitor of potassium channels, 1 µM), IBTX (Large conductance Ca^{2+} activated K^+ channel blocker or voltage-sensitive potassium channels, 10 µM) and Glib (K_{ATP} channels inhibitor, 0.44 µM) 30 min before the addition of ALF. Experiments were performed in the presence of indomethacin (n=4 to 6 different experiments; *P<0.05 vs. Control).

vasodilation and also to show the implication of PDEs inhibiton activity by this fraction of *A. leiocarpa* in an organ model.

Thus, in endothelium-denuded coronary artery ring, vasoconstriction was induced by the thromboxane A2 analogue U46619. The thromboxane A2 (TXA2) was a pharmacological agonist known to be a potent constrictor of artery smooth muscle as well as an inducer of platelet aggregation but also a major cyclooxygenase-dependent endothelium-derived contracting factor (Fu et al., 2017; Minuz et al., 2018; Xu et al., 2018). Because thromboxane A2 was an unstable component, the stable U46619 was

used to contract the coronary arteries rings in the presence or absence of active substances. Indeed, the U46619 was a full TXA2 receptor agonist that induced a susbtained contraction in porcine coronary arteries rings by an influx of Ca^{2+} transmembrane leading to the increase of intracellular Ca^{2+} (Hanasaki et al., 1988; Han et al., 1995; Cogolludo et al., 2003; Hahnenkamp et al., 2004; Sinharoy et al., 2017). Other studies have been also indicated that the important mechanism by which TXA2 elicits its role in pulmonary vasoconstriction was through the involvement of an increase in $[Ca^{2+}]_i$ in the smooth muscle cells (Chakraborti et al., 2009).

The present findings showed that ALF had significantly reduced the U46619-induced vasoconstriction in porcine coronary artery rings without endothelium and interestingly, this effect was in a concentration-dependent manner (Figure 1). For that, ALF effect could be explained by a possible decrease in $[Ca^{2+}]_i$ release in the vascular smooth muscles cells (VSMC) since U46619 was known to induce vasoconstriction in vessels by activation of PLC via Gaq, followed by an increase in intracellular free calcium concentration through an inositol-3-phosphate stimulation sensitive receptors (Hirata et al., 1991; Somlyo and Somlyo, 2000; Bhattacharya et al., 2005).

In order to determine the possible implication of Ca^{2+} decrease in ALF-induced vasodilation effect in vascular system, the effect of SNP was investigated in denuded artery rings pre-incubated with different concentrations of ALF and contracted with U46619 (Figure 2). In this study, results showed that in the functional vascular study using wire myograph and SNP which acts directly on VSMC, that ALF acutely displays an increase in endothelium-independent relaxation in a concentration-dependent manner compared to the control.

In the literature, the SNP (a NO donor) was a well-known arterial and venous vasodilator used in clinical practice to lower blood pressure and usually used to increase the endothelium-independent vasodilatation effect by a cGMP independent mechanism (Otsuka et al., 1988; Hottinger et al., 2014; Basrali et al., 2015). Indeed, several studies demontrated that in vascular smooth muscle, the NO has activated the soluble guanylate cyclase (sGC) that stimulate the production of cyclic GMP, which induced a reduction in the concentration of cytosolic Ca^{2+} with consequent vascular relaxation (Rapoport and Murad, 1983; Hottinger et al., 2014; Zhao et al., 2015; Montfort et al., 2017). Because ALF has enhanced SNP effect suggested that ALF and SNP may have possible synergetic actions to promote cGMP accumulation and Ca^{2+} decreased in VSMC.

Moreover, the endothelium-independent relaxation to isoproterenol (a β-adrenoceptor agonist) was enhanced by the presence of ALF which was in a concentration-dependent manner (Figure 4). Because isoproterenol was known to reduce VSMC vasoconstriction state by stimulating intracellular cAMP level leading to vasodilation suggested a possible synergetic action between ALF and isoproterenol vasorelaxant pathway (Mokkapatti et al., 1998; Xu et al., 2006; Townsend et al., 2012; Ruiz-Medina et al., 2018). The present findings are in accordance with those of other authors which have shown that extract of *Thymus linearis* Benht significantly reduced the contraction effect of U46619 in endothelium-denuded porcine coronary rings in concentration dependent (Alamgeer et al., 2018). In addition, the extract of this plant has significantly improved the effect of isoproterenol, which indicated a certain implication of the cAMP relaxation pathway that was in line of those of ALF. Likewise, the ALF effect might also be due to other mechanisms of actions such as the PDEs inhibition pathway and confirm our previous study (Belemnaba et al., 2013). In fact, the previous study indicated that ALF endothelium-independent effect might be explained by a stimulation of the K^+, Na^+/K^+-ATPase channels but also via inhibition of PDEs activity.

The inhibition of PDEs fosters the accumulation of second messengers according to their family. Under normal physiological conditions, PDE1 was activated by the Ca^{2+}-calmodulin complex. On the one hand, PDE2 and PDE1 have used cAMP and cGMP as substrates for their activity following a decrease in the two second messengers normally responsible for the vasodilatation of VSM (Lugnier, 2006, 2011; Keravis and Lugnier, 2012; Bobin et al., 2016). On the other hand, the PDE5 have used cGMP as substrate and their inhibition leads specifically to the accumulation of cGMP at the smooth muscle level (Lugnier, 2006). In the present findings, the results have shown that the effects of SNP in the presence of nimodipine (PDE1 specific inhibitor) and EHNA (PDE2 specific inhibitor) were slightly shifted to the left but not significantly different as compared to the control.

These results suggest that, in the presence of EHNA, cGMP accumulated by the action of SNP on sGC reactivates PDE2 present in VSM which degrades this substrate thus reducing the effect of sGC activator. However, nimodipine did not significantly improve the effect of SNP compared to the control and its effect in the presence of EHNA. These results indicate a possible involvement of PDE5 which degrade the cGMP produced by the SNP in the case of PDE2 inhibition by EHNA; insofar as in the presence of DMPPO, this effect was significantly different from the control (Figure 3). Thereby, these results corroborate those of others studies in which, in rat lungs precontracted with U46619, the DMPPO amplifies the vasodilator effects of sodium nitroprusside (Eddahibi et al., 1998).

Furthermore, in the present study, the results showed that the effect of isoproterenol on rings pre-incubated with ALF 3 µg/mL is comparable to those obtained in the presence of nimodipine and DMPPO (Figure 5). On the

other hand, this effect in the presence of ALF (10, 30 or 100 µg/mL) is improved and comparable to that obtained with rings pre-incubated with cilostamide (PDE3 inhibitor, Figure 6). Thus, at high concentrations, ALF would also inhibit PDE3 to ensure its vasodilator effect.

Because PDE3 was well known to have a high affinity for cAMP and hydrolyzes cAMP with a rate 10-fold greater than for cGMP hydrolysis despite its capacity to hydrolyze both cAMP and cGMP, this study suggests a possible increase of cAMP and cGMP in VSM after ALF treatment (Beavo, 1995). These results are in line with other studies performed in denuded rat aorta which led to vasodilation and this effect was enhanced by the cilostamide (PDE3 inhibitor) and by the combinaison of SNP plus DMPPO (Delpy et al., 1996; Cui and Green, 2003).

Moreover, the large-conductance calcium-activated potassium channels (BK_{Ca}) were an important potassium ion channels in the VSMC membrane. A depolarization of VSMC membrane potential was followed by an increase of BK_{Ca}, leading to the increase of the intracellular potassium efflux responsible of membrane hyperpolarization. Since then, L-type-calcium channel opening was reduced and was followed by the intracellular calcium concentration which decreased, responsible to vasodilation (Qian et al., 2017). In this study, results showed that ALF has no significant effect on denuded rings pre-incubated with IBTX indicating that BK_{Ca} has not been involved in ALF endothelium independent vasodilation effect.

Likewise, investigation of ALF effect with the K_{ATP} channels inhibitor (Glibenclamide), showed that this effect was slightly but not significantly enhanced compared to the control. That result suggests that K_{ATP} pathway appears not to be involved in the ALF vasodilation process (Figure 7). The same observation was obtained with the non-selective K^+ channel blocker (TEA) claiming that ALF would induce its effect by preferentially inhibited by the PDE1, 3, and 5 activity than K^+, K_{ATP} and BK_{Ca} channels involved.

Conclusion

The present findings demonstrated that ALF obtained from barks of trunk of *A. leiocarpa* was able to induce endothelium-independent vasodilation effect in denuded porcine coronary arteries rings pre-contracted with U46619. This effect might be due to a possible decrease in $[Ca^{2+}]_i$ and the reduction of the membrane hyperpolarization. In addition, ALF endothelium-independent effect was more due to its capacity to inhibit PDE1, 3 and PDE5 in the VSMC but not the inhibition of the PDE2. Moreover, it also appears that the implication of openned channels (BK_{Ca}, K^+ and K_{ATP}) was too lessier than those of the inhibition of PDEs and the Ca^{2+}-decrease in vessels. Then, the efficacy for acute and chronic usage of ALF needs to be investigated in *in vivo* model.

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CONFLICT OF INTERESTS

The author has not declared any conflict of interests.

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