Original Research Article

Endothelium-Independent Vasorelaxant Effects of Anthocyanins-Enriched Extract from *Odontonema Strictum* (Nees) Kuntze (Acanthaceae) Flowers: Ca^{2+} Channels Involvement

ABSTRACT

Aims: We aimed in this study to investigate the mechanisms of the vasorelaxation effect caused by the anthocyanins-enriched extract of *Odontonema strictum* flowers.

Study Design: Anthocyanins-enriched extract of *Odontonema strictum* flowers and vasorelaxantes activities of mice aortic rings.

Place and Duration of Study: The flowers of *Odontonema strictum* (Nees) Kuntze (Acanthaceae) were collected in January 2015 at the "Institut de Recherche en Sciences de la Santé (IRSS)" experimental station in Ouagadougou. The experiments were conducted in October - November 2018 at the department of Medicine and Traditional Pharmacopeia-Pharmacy (MEPHATRA-PH)/IRSS.

Methodology: The extract was enriched in anthocyanins using Amberlite XAD-7 non-ionic resin column. The vasorelaxant activity of anthocyanins-enriched extract of *O. strictum* flowers (OSF) was tested using isolated organ-chamber technique with mice aorta rings.

Results: OSF showed concentration-dependent relaxant effects on mice endothelium intact or denuded aortic rings pre-contracted with U46619 (10^{-7} M) and KCl (80 mM). OSF induced relaxation in the mice aortic rings by stimulating smooth muscle cells. The vasorelaxant effect of OSF (10-1000 µg/mL) was similar in endothelium-intact and endothelium-denuded aortic rings. The maximum relaxant effect was 93.78 ± 4.69% and 92.30 ± 3.19% for endothelium-intact and endothelium-denuded aortic rings, respectively. Moreover, after incubation of the aorta rings with OSF (400 µg/mL) or vehicle (0.02% of DMSO) in PSS, OSF blocked the contraction through mechanism involving inhibition of $CaCl_2$ and U46619 effect.

Conclusions: The present study provides a pharmacological evidence for the antihypertensive medicinal use of *Odontonema strictum* by highlighting its vasorelaxant activity.

Keywords: *Odontonema strictum*; flowers; endothelium-independent; vasorelaxant; calcium channels

1. INTRODUCTION

In the past decade, epidemiological studies have shown that cardiovascular diseases are the main cause of death and disability worldwide [1, 2]. In Africa, cardiovascular diseases (CVD) have reached nearly epidemic proportions. Indeed, high blood pressure is the major determinant of mortality related to cardiovascular disease, cerebrovascular disease, and stroke [3]. High blood pressure is a major cause of deaths in developing countries, accounting for about 8-9 million of deaths compared to about 3-5 million in developed countries [4]. The pathophysiological mechanism behind this disorder is multifactorial and include oxidative stress, inflammation, renin-angiotensin system and autoimmune vascular dysfunction [5-7]. Hypertension is characterized by a chronic elevation of arterial blood pressure (superior or equal to 140/90 mmHg), in which abnormally increased vascular tone plays a major role in the maintenance of high blood pressure [2, 8].

Although, conventional drugs continue to be developed against hypertension, they do not fully manage this condition. In such context, 80% of the population resort to traditional medicine for their health care, including hypertension [9]. In addition, natural drugs are another alternative to synthetic drugs [10]. Natural products represent an extremely valuable source for production of news chemicals entities for the treatment of emerging diseases, since they represent structures selected by evolutionary mechanisms over a period of millions of years through an adaptation according to time and climate [1, 11]. Previous studies reported the use of plants in traditional medicine to treat various diseases including cardiovascular disease. It is very important to screen plants or plants extracts for the treatment of diseases such as hypertension [7]. Among the available plants, *Odontonema strictum* (Nees) Kuntze (Acanthaceae) a decorative plant of Latin America, is known to be traditionally used for the treatment of arterial hypertension [12]. Authors have reported its antihypertensive/hypotensive and vasorelaxation effects on rat and pig heart coronary arteries respectively [13]. These authors have shown through pharmacological tests that the aqueous, alcoholic and ethyl acetate extracts from the plants leaves possess antihypertensive and vasorelaxant properties. Following this work, C-heteroside flavonoids and

O-heteroside flavonoids were isolated from the leaves of *Odontonema strictum* [12]. These leaves extracts have shown antioxidant properties [9, 14]. *Odontonema strictum* leaves and flowers contain carbohydrates, saponins of flavonoids, glycosides, tannins, steroids and terpenoids as well as Stigmasterol and β-Sitosterol [15].

All these pharmacological investigations were mainly focused on the *Odontonema strictum* leaves. In addition, anthocyanins are the main phenolic compounds involved in the color of flowers and they also possess physiological activities such as antioxidative, antimutagenic and antihypertensive properties [16]. No data is available on *Odontonema strictum* flowers. Therefore, this study has been undertaken to assess the antihypertensive efficacy of *Odontonema strictum* flowers and to characterize its vasorelaxant activity, as a potential mode of action.

2. MATERIAL AND METHODS

2.1. Plants Material

Odontonema strictum flowers were collected in January 2015 at "Institut de Recherche en Sciences de la Santé" experimental station in Ouagadougou (GPS coordinates N 12°22.161', W 001°29.088'). The plant was properly identified, and a voucher specimen (HNBU 8702) was deposited in the herbarium of the "Département Environnement et Forêt / Centre National de la Recherche Scientifique et Technologique" (DEF/CNRST), Ouagadougou, Burkina Faso [13]. The plant material (flowers) was washed carefully before drying at lyophilization and powdered into a fine powder in a blender.

2.2. Extract enriched with anthocyanins

We mixed 100 mL of n-hexane (≥ 99.7%, Sigma-Aldrich) with 10 grams of *Odontonema strictum* flowers powder to remove fats and lipid compounds; then the solid phase was macerated with 100 mL of methanol (≥ 99.9%, Sigma-Aldrich) for 24 hours at 4 °C in the refrigerator. The maceration process with methanol was repeated twice and the filtrated extracts were pooled and concentrated under vacuum to dryness under 40 °C. The dried extract was solubilized in 5 mL of methanol (0.5% HCl) for further purification.

Further enrichment of anthocyanins was obtained with 5 mL of methanol (0.5% HCl) using Amberlite XAD-7 (Sigma Life Sciences), non-ionic resin column. Amberlite XAD-7 column was

initially washed with 0.5% HCl to remove free sugars and non-aromatic compounds. This resin adsorbed the aromatic compounds including anthocyanins, whereas sugars and non-aromatic compounds were eluted by washing with acidified water (0.5% HCl). The adsorbed anthocyanins were eluted by acidified methanol (0.5% HCl). The pooled methanolic was concentrated on a rotavapor under vacuum at 40 °C to obtain dried powder.

2.3. Animals

Male mice (6-8 weeks old) Naval Medical Research Institute (NMRI) were obtained from the pet Shop of IRSS, Ouagadougou and exposed to daily light-dark 12 hours cycle with free access to proteins enriched pellet (29%) and water. They were maintained in controlled temperature room of 22-25°C.

2.4. Preparation of mice thoracic aortic rings for isometric tension recording

The method used has been previously described [17, 18]. Briefly, mice were euthanized and thoracic aortas were excised, cleaned from fat tissue and cut into 2 mm length-rings. The aorta was immersed in physiological salt solution (PSS). The composition of PSS (in mM) was 130 NaCl, 14.9 $NaHCO_3$, 3.7 KCl, 1.2 $MgSO_4$ $7H_2O$, 1.6 $CaCl_2$ $2H_2O$, 1.2 KH_2PO_4, and 11 glucose), pH 7.4. The PSS was continuously kept at 37 °C and aerated with a pneumatic bubbling. Isolated mouse aortic rings were suspended in organ chambers containing PSS and placed between 2 tungsten stirrups. Aortic rings were stretched with a passive wall tension of one (01) g while an equilibrium period of 60 min was allowed during which it was washed every 20 min. After stabilization, the aorta was contracted by addition of KCl (bath concentration of 80 mM). Subsequent to washing increasing concentrations (10^{-9} - 3.10^{-7} M) of a thromboxane A2 analogue agonist (U46619) were cumulatively added and the maximal tissue's tension was recorded. Changes in tension were recorded via isometric force transducers connected to a data acquisition system. The endothelium integrity was checked by the ability of ACh (10^{-5} M) to induce more than 80% relaxation in U46619-contracted aorta rings. When necessary, the endothelium layer was removed, by gently rubbing inside the lumen using forceps. The endothelium-denudation was considered effectively removed when ACh (10^{-5} M) caused less than 10% relaxation. Ca^{2+}-free PSS was prepared by removing $CaCl_2$. The thromboxane A2 analogue, 9,11-dideoxy-9α,11α-methanoepoxy $PGF_{2\alpha}$ (U46619, Merck Chemicals Ltd, Nottingham, UK) and the NO• synthase

inhibitor, N(ω)-nitro-L-arginine methyl ester (L-NAME, Sigma-Aldrich, 100 µM) and the non-specific cyclooxygenase inhibitor, indomethacin (Indo, Sigma-Aldrich, 10 µM) were selected for the experiments. The enriched anthocyanin extract of *Odontonema strictum* (OSF) flowers induced vasorelaxant effects in a concentration-dependent manner (10 - 1000 µg/mL) on mice aortic rings. These aortic rings intact and denuded of the endothelium are previously contracted with U46619 (10^{-7} M) or KCl (80 mM) in PSS.

The effect of OSF (400 µg/mL) on extracellular Ca^{2+} influx was studied in Ca^{2+}-free PSS. After equilibration of rings in Ca^{2+}-free PSS containing 80 mM KCl, cumulative concentrations of $CaCl_2$ were added (10^{-5} - 10^{-2} M, respectively) with preincubation of OSF in organ bath. The $CaCl_2$ concentration-dependent maximum contraction of the endothelium denuded aortic rings with KCl (80 mM) in Ca^{2+}-free PSS was expressed as 100% for the curve constructions. The aortic rings were measured after each experiments.

To study the relationship between the intracellular Ca^{2+} release inhibition and the OSF-induced relaxation, endothelium denuded aortic rings were incubated with OSF (400 µg/mL) or vehicle (0.02% of DMSO) in PSS, then the aortic rings were measured after experiment to report each maximum contraction to the size of the ring.

2.5. Statistical analysis

The experimental values were calculated by considering the maximum contraction produced by U46619 of each segment equal to 100%. The baseline tension before addition of U46619 was considered as 0%. The raw data have been normalized to the control (vehicle). Concentration-response curves were fitted using a nonlinear interactive fitting program (Graph Pad Prism 5.0; GraphPad Software, San Diego, CA), and two pharmacological parameters were obtained: the maximal effect generated by the agonist (Emax) and pD2 (-log EC_{50}) [EC_{50} is a concentration of agonist producing 50% of the maximum response]. Statistical comparisons were performed using one-way ANOVA or two-way ANOVA. Post hoc test was performed using Bonferroni's test analysis to compare all the groups. A p-value less than 0.05 was considered as statistically significant.

3. RESULTS

3.1. Effect of anthocyanins extract of *Odontonema Strictum* flowers (OSF) on U46619-induced contraction of endothelium-intact mice aortic rings

The effect of OSF (10 - 1000 µg/mL) extract on endothelium-intact aortic rings contraction is concentration dependent. In Fig. 1A, OSF effect was compared to that of the control, while Figs. 1B, C shows OSF vasorelaxant effect on the nitric oxide ($NO^{\bullet}$) synthesis pathway and prostacyclin (PGI_2) pathway in endothelium-intact aortic rings (Figs. 1B, C). Incubation with L-NAME (100 µM, an eNOS inhibitor) and the combination of L-NAME and indomethacin (10 µM, a non-selective COX inhibitor), did not affect OSF relaxation effect on endothelium-intact aortic rings pre-contracted by U46619. In the absence and presence of L-NAME or indomethacin or both of them, the maximal relaxant effects (Emax) of OSF on endothelium-intact aortic rings were respectively 93.78 ± 4.69%, 91.67 ± 4.31%, 91.99 ± 2.18% and 89.45 ± 5.99%. The pD2 of the different relaxation conditions are presented in table.

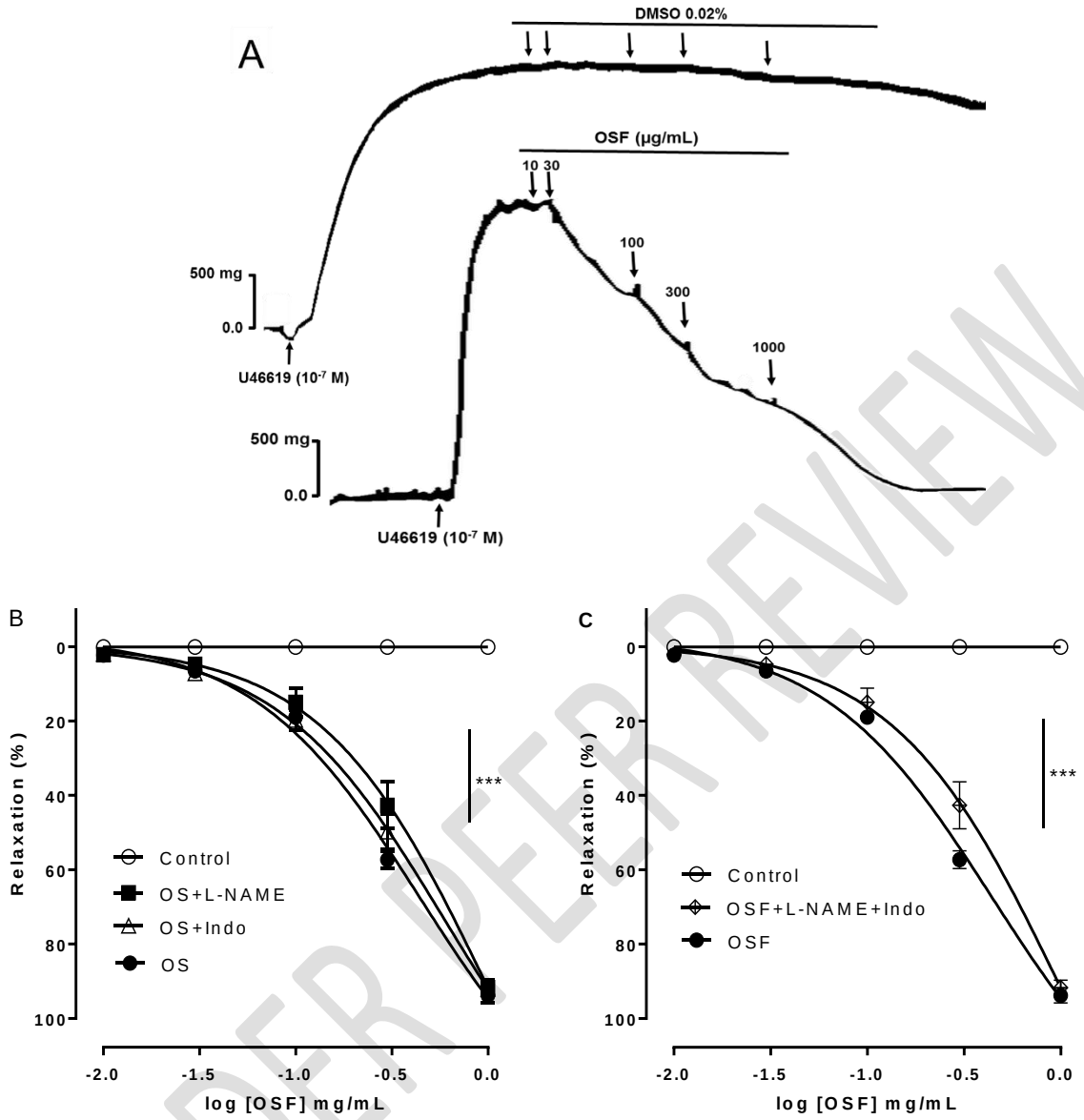

Fig. 1. Cumulative concentration-response curves of OSF on endothelium-intact aortic rings pre-contracted with U46619. OSF effect (illustration, A) in the absence (control) or presence of L-NAME, Indomethacin (B), or both (C). Values are expressed as mean ± SEM (n = 5-6). ***$p < 0.001$ *vs.* Control

3.2. Anthocyanins-enriched extract of *Odontonema strictum* flowers effect on U46619-induced contraction of endothelium-intact or endothelium-denuded aortic rings

The ability of OSF extract to relax vascular tone was assessed using mice artery rings contracted submaximally with thromboxane A2 agonist receptor, U46619. We investigated the concentration-dependent vasorelaxant effect of OSF (10 - 1000 µg/mL) on endothelium-intact

and endothelium-denuded aortic rings. The magnitude of endothelium (intact or denuded) relaxation is a function of OSF concentration (Fig. 2). However, the functional removal of the endothelium did not modify OSF-induced relaxation in U46619-precontracted rat thoracic aorta rings. The pD2 of OSF in presence and in absence of endothelium are recorded in the table. The maximal relaxant effect was 93.78 ± 4.69% and 92.30 ± 3.19% for endothelium-intact and endothelium-denuded aortic rings, respectively.

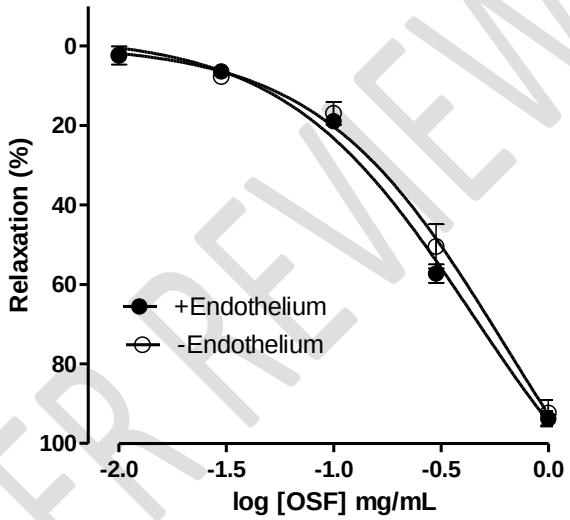

Fig. 2. Vasorelaxant response induced by OSF on U46619-induced pre-contractions in endothelium-intact or -denuded rat aortic artery isolated rings. Values are expressed as mean ± SEM (n = 5-6)

Table: Vasorelaxant parameters of OSF (pD2) during exposure of mice aortic rings contracted with U46619 in the absence and presence of L-NAME and indomethacin

Substance administered	pD2 (mg/mL)
U46619+OSF	0.24 ± 0.03***
L-NAME+U46619+OSF	0.22 ± 0.04***
Indomethacin+U46619+OSF	0.21 ± 0.04***
L-NAME+ Indomethacin+U46619+OSF	0.19 ± 0.05***
Denuded endothelium+U46619+OSF	0.22 ± 0.04***
Control (0.02% of DMSO)	0.0

*** $p < 0.001$ *vs.* Control

3.3. OSF effect on KCl (80 Mm)-induced contraction of endothelium denuded mice aortic rings

We have also investigated the effect of OSF on aortic rings without endothelium precontracted by depolarization with high concentration of K^+. The results have shown that OSF had relaxed KCl (80 mM)-precontracted aortic rings in a concentration dependent manner (Fig. 3). The maximal relaxant effect was 99.72 ± 2.32% and pD2 = 0.12 ± 0.06 mg/mL (Table).

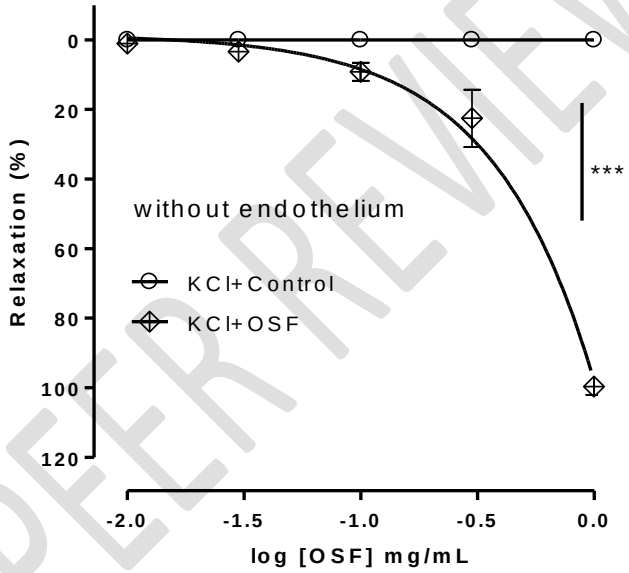

Fig. 3. Concentration-response curves of OSF on KCl (80 mM) induced contraction in mice endothelium denuded aortic artery rings. Values are expressed as mean ± SEM (n = 4). ***$p < 0.001$ *vs.* Control

3.4. Effect of anthocyanins-enriched extract of *Odontonema Strictum* flowers on extracellular Ca^{2+}-induced contraction

To investigate the role of extracellular Ca^{2+} influx, cumulative addition of $CaCl_2$ (10^{-5} - 10^{-2} M) in Ca^{2+}-free PSS medium containing KCl (80 mM) have been realized on endothelium-denuded aortic rings. As compared to control (0.02% of DMSO), preincubation rings with OSF (400 µg/mL) strongly inhibited Ca^{2+}-induced contraction in aorta rings ($p < 0.001$). The maximal contractions induced by $CaCl_2$ (10^{-2} M) were 704.32 ± 50.43 mg/mm and 44.24 ± 29.46 mg/mm in the absence and presence of OSF (400 µg/mL), respectively (Fig. 4).

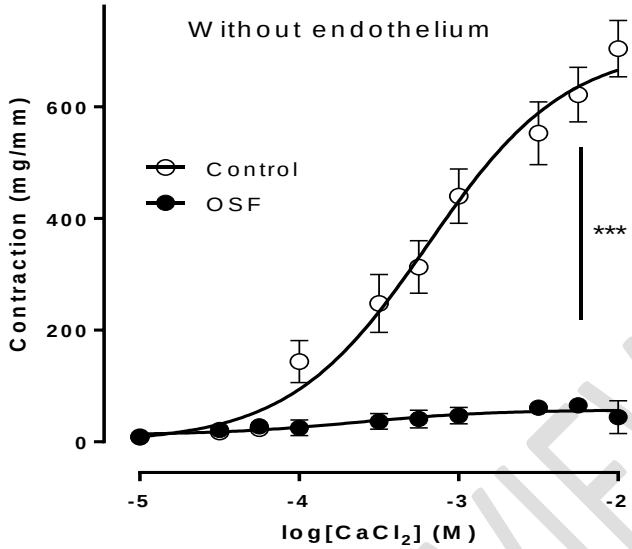

Fig. 4. OSF Inhibitory effect on contraction induced by extracellular Ca^{2+} in endothelium denuded of mice thoracic aortic rings in PSS containing KCl 80 mM. Values are expressed as mean ± SEM (n = 5). * *p* < 0.001 *vs.* Control.**

3.5. Effects of anthocyanins-enriched extract of *Odontonema strictum* flowers on mice aortic rings by cumulative U46619

The preincubation of OSF (400 µg/mL) for 20 min was able to inhibit in a concentration dependent manner, the contractile response induced by U46619 on denuded-endothelium rings. The concentration response curves was significantly rightward with OSF inhibition effect (Fig. 5). The maximal contractions induced by U46619 (3.10^{-7} M) were 1284.34 ± 75.17 mg/mm and 490.88 ± 90.53 mg/mm in the absence and presence of OSF (400 µg/mL), respectively.

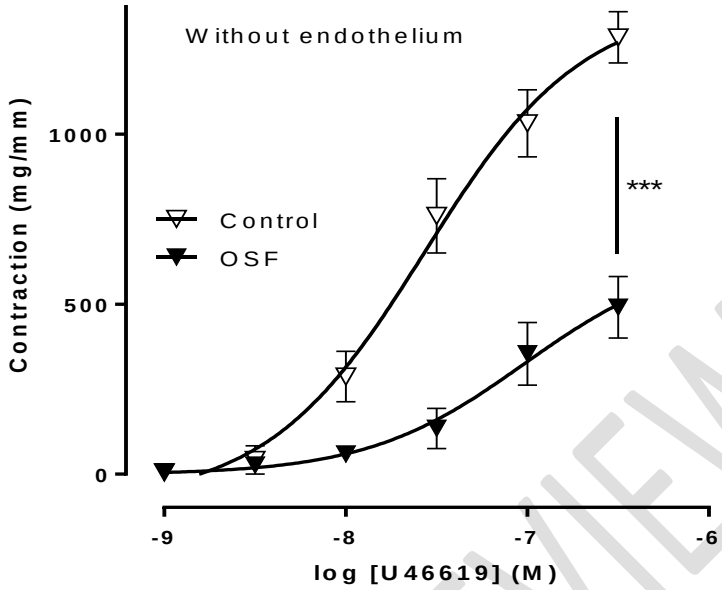

Fig. 5. Inhibitory effect of OSF on contraction induced U46619 in endothelium-denuded of mice thoracic aortic rings in PSS. Values are expressed as mean ± SEM (n = 5). *** $p < 0.001$ *vs.* Control.

4. DISCUSSION

Vascular smooth muscle vasoactivity is regulated by circulating factors from the endothelium, neurotransmitters and hormones [19]. The vasorelaxing effect is generally classified as endothelium-dependent or independent depending on the function of the endothelium. Endothelial cells secrete vasorelaxant substances such as nitric oxide (NO•), prostacyclin (PGI_2) and endothelium-derived hyperpolarizing factor (EDHF) to regulate vascular smooth muscle tone and endothelial-derived contracting factors such as endothelins, angiotensin II, prostanoids derived from cyclooxygenase and superoxide anions [18, 20]. The present study showed that the anthocyanins-enriched extract (prepared from *Odontonema strictum* flowers (OSF) inhibited U46619, KCl, $CaCl_2$ contractile responses in mice aorta rings; the response is dose dependent. Indeed, many studies have reported that plant extracts exert vasculo-protection via their ability to induce the relaxation of blood vessels by a mechanism dependent on NO• and PGI_2 release [21-23]. Thus, we aimed at checking the first hypothesis. The inhibitory effect of OSF on U46619-induced contraction was not affected either in the presence of L-NAME or indomethacin or in the combination of L-NAME plus Indomethacin. Relaxant effect of NO• is mainly due to an increase in cyclic guanosine monophosphate (cGMP) [24, 25]. Blocking this NO•/cGMP pathway with L-NAME and indomethacin as a nonselective inhibitor of COX had no effects on the vasorelaxant

effect of the extract on U46619-induced contractions. Thus, the relaxant effect of the OSF is independent of production NO• or prostacyclin. OSF also decreased U46619-induced contractions in endothelial denuded aortic rings, as it mediated the same relaxation at similar concentration as in the intact-endothelium. These results suggest that the relaxant effect has been exerted on the vascular smooth muscle cells and not a lesser involvement of EDHF. Interestingly, the endothelium-independent mechanism of OSF could help alleviate high blood pressure associated with endothelial dysfunction by acting directly on smooth muscle. Indeed, authors have shown that plant extracts could act directly on the vascular smooth muscle to induce vasodilation [20, 26, 27].

In addition, contraction and relaxation of vascular smooth muscle cells are regulated by Ca^{2+} entry from the extracellular space through Receptor-Operative Ca^{2+} Channels (ROCCs) or Voltage-Dependent Ca^{2+} Channels (VDCCs) in the cytoplasmic membrane, through Ca^{2+} release from intracellular Ca^{2+} stores (sarcoplasmic reticulum) by activation of 1,4,5 triphosphate inositol (IP_3) and ryanodine receptors (RyR), protein kinase C (PKC) activation, and a Ca^{2+} sensitization mechanism [28]. Moreover, previous reports have shown that the smooth muscle cells contraction elicited by KCl (high K^+, >30 mM) mainly results from the influx of extracellular Ca^{2+} induced by depolarization of the cells membrane and subsequent opening of the voltage-dependent slow Ca^{2+} channels (VDCCs) [29]. We therefore assessed whether OSF extract could inhibit calcium entry activated by the VDCCs. We looked at OSF effect on the contraction in response to KCl^- depolarization. As a result, OSF relaxed vasoconstriction induced by KCl in rings. We also demonstrated that OSF caused vasorelaxation of KCl-induced contraction in mice isolated aortic ring through possible inhibition of VDCCs. Interestingly, OSF inhibited also dramatically the contraction of endothelium- denuded aortic rings induced by Ca^{2+} supplementation in in Ca^{2+} free - PSS containing KCl (80 mM). These results suggested that OSF have blocked both ROCCs and VDCCs involved in the vasodilatation activity. Indeed, the influx of extracellular Ca^{2+} is mainly regulated by receptor operated calcium channels (ROCCs) or VDCCs [25, 29-31].

The thromboxane A2 analogue agonist (U46619) acts by stimulating the production of phospholipase C (PLC). Afterwards, PLC produce diacylglycerol (DG) and IP_3, and subsequently DG activates the light chain of myosin through activation of protein kinase C (PKC), and IP_3 induces Ca^{2+} release from the sarcoplasmic reticulum by opening IP_3 receptors and by Ca^{2+} influx

through ROCCs [3, 20, 21, 32, 33]. To verify the involvement of this pathways in the OSF vasodilation effect, the rings were preincubated with OSF before the cumulative of U46619 (10^{-9} - 3.10^{-7} M). The results showed that OSF significantly reduces this agonist-induced contraction in mice aorta. Thus, OSF effect could be due to the inhibited the IP_3 and/or ryanodine receptor-dependent release of intracellular Ca^{2+}. It can also reduce DG-PKC dependent myosin light chain kinase activity, and/or block ROCCs to decrease intracellular Ca^{2+} and relax the mice aorta as reported previously [20, 25, 31]. In order to better determine the mechanisms of action on the muscle cell, the use of specific inhibitors would be necessary for future investigations.

According to the published literature, the presence of phytochemical components in *Odontonema strictum* flowers such as saponins of flavonoids, tannins, steroids and terpenoids could explain the vasodilation effects of OSF [15]. Indeed, many authors have demonstrated that flavonoids (saponins) [34], tannins [35], steroids and terpenoids [36] have endowed vasodilator property. Furthermore, these phytochemical groups have antioxidant properties [9, 14, 37] that could provide a vascular protection effect by neutralizing reactive oxygen species which are known to have constrictive effects. Oxidative damage could cause endothelial cells injuries and deleterious vasodilator effects. It has been shown that antioxidant compounds could modify molecular events towards an improvement of the endothelium function, and therefore play an important role in the prevention of CVD [38, 39].

5. CONCLUSION

The present study has provided a pharmacological evidence for the vasorelaxant activity of *Odontonema strictum*. This effect is mediated by the endothelium-independent pathways including the blockade of the extracellular calcium influx and intracellular calcium-release. Therefore, this study supports the evidence that the extract of OSF could be a valuable alternative for the treatment of hypertension in Burkina Faso.

CONSENT

It is not applicable.

ETHICAL APPROVAL

The laboratory experimentation was carried out according to the experimental protocols approved and validated by the MEPHATRA-PH/IRSS laboratories and meeting the international standards in this field. The protocol was conducted in accordance with the institutional Ethics Committee for Animals protection regulations (directive 2010/63/EU on protection of animals used for scientific purposes). Ethical approval code: 2010/63/EU, Date of approval: 20 October 2010.

COMPETING INTERESTS

The authors declare that there is no conflict of interests to disclose regarding the publication of this paper.

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