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RESEARCH ARTICLE

Antioxidant activity and vasorelaxant effect of *Marrubium multibracteatum* via receptor-activated calcium channels and nitric oxide pathways in rats

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Abstract

According to several studies, antihypertensive drugs have side effects on human health. Therefore, natural products are also being investigated as alternatives to these drugs. For this reason, the present study aimed to examine the vasorelaxant properties of *Marrubium multibracteatum* aqueous extract (MMAE) on isolated rat aortic rings. The antioxidant potential of MMAE was also evaluated. The vasorelaxant activity of MMAE was assessed on aortic rings precontracted with epinephrine (EP, 10 µM) and potassium chloride (KCl, 80 mM). The antioxidant potential of MMAE was determined using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay. In addition, the extract underwent phytochemical screening. The results showed that cumulative concentrations of MMAE (0.75 – 1.50 mg/mL) induced a vasorelaxant effect on epinephrine-precontracted aortic rings via the nitric oxide (NO) pathway and inhibited extracellular Ca^{2+} entry through receptor-operated calcium channels (ROCCs). However, the same concentrations of MMAE exhibited only minimal vasorelaxant effects on KCl (80 mM)-precontracted aortic rings. In contrast, MMAE demonstrated significant antioxidant activity. These findings suggest that MMAE exhibits both potent vasorelaxant and antioxidant properties, likely due to its phytochemical composition, particularly the presence of polyphenols, flavonoids and tannins. Therefore, this extract may represent a promising natural alternative to certain synthetic antihypertensive drugs.

Keywords: antioxidant activity; *Marrubium multibracteatum*; nitric oxide; receptor-operated calcium channels; vasorelaxant

Introduction

Hypertension is a major global public health problem. As a silent disease, it is the leading preventable cause of cardiovascular disease and stroke worldwide. It can damage vital organs such as the heart, brain and kidneys and lead to severe complications, including myocardial infarction, heart failure and stroke (1). A recent cross-sectional study conducted between September and December 2024 in eastern Morocco among 163 adult women (mean age: 43.3±13.3 years) reported a hypertension prevalence of 26.4 % (2). According to the World Health Organisation (WHO), the number of adults with hypertension has nearly doubled globally over the past 3 decades, rising from 650 million in 1990 to 1.3 billion in 2019. The growing burden of elevated blood pressure has resulted in an estimated 10.8 million preventable deaths annually and approximately 235 million years of life lost or lived with disability (3). Vascular tone and vasodilation play a critical role in controlling hypertension. At rest, the arterial vasculature is in a state of vasoconstriction (vascular tone). To maintain normal blood pressure, physiological dilation in response to increased blood flow requires normal endothelial function. The absence of normal vasorelaxant function and the inability of arteries to dilate properly in response to increased blood flow, whether in a systemic or regional vascular bed, are consequences of endothelial dysfunction of vascular smooth muscle cells, resulting in hypertension (4). Conversely, a drop in blood pressure can impair the functioning of organs and cellular systems by reducing blood flow and oxygen delivery to peripheral tissues (1).

The treatment of hypertension commonly involves antihypertensive drugs that are considered appropriate components of first-line therapy. These include angiotensin II type 1 (AT1) receptor blockers (sartans), dihydropyridine calcium

channel blockers, angiotensin-converting enzyme (ACE) inhibitors, beta-adrenergic receptor blockers and thiazide-type diuretics (5). However, these drugs are associated with various side effects, such as renal dysfunction, fatigue, dry cough and abnormal heart rate (6). In contrast, some plant-derived compounds have shown promising antihypertensive effects. For example, an aqueous extract of oak moss has been reported to exhibit antihypertensive activity through the soluble guanylate cyclase cyclic GMP (sGC-cGMP) pathway in rats with L-NAME-induced hypertension (7). Therefore, natural products are also being investigated as an alternate source for antihypertensive drugs.

Marrubium multibracteatum (M. multibracteatum) belongs to the Lamiaceae family. It contains marrubiin, a labdane-type diterpene characteristic of the genus, along with various phenolic compounds (8). Species of this genus are used as food flavourings and for medicinal purposes (9). Numerous studies indicate that *Marrubium vulgare* possesses strong antioxidant activity, which may make it useful in the treatment of liver diseases, diabetes mellitus and cancer. Furthermore, it has been reported to exhibit antihypertensive, anti-inflammatory, lipid-lowering and antibacterial properties, particularly against parasites, fungi, herpes simplex virus and gram-positive bacteria (8). Several species of the *Marrubium* genus demonstrate antioxidant, antihypertensive and anti-inflammatory activities. However, no data are currently available regarding the biological activities of *M. multibracteatum*. This study aimed to evaluate the vasodilatory properties of the aqueous extract obtained from the aerial parts of *M. multibracteatum* and to elucidate its potential mechanism of action on isolated rat thoracic aorta. Additionally, the antioxidant activity of the extract was assessed using the DPPH free radical scavenging assay and a phytochemical screening was performed. All data generated or analysed during this study are included in this published article.

Materials and Methods

Plant material and preparation of the aqueous extract

In March 2023, fresh aerial parts of *Marrubium multibracteatum* were collected and air-dried. The plant was obtained from a resident of Mount Ayachi, located in the eastern region of the Moroccan high atlas. The plant material was authenticated by members of the Ethnopharmacology and Pharmacognosy team at the Faculty of Science and Technology of Errachidia. A voucher specimen (MM 2024) was deposited in the herbarium of the same faculty. The fresh plant-to-powder ratio was 5:1 (i.e., 5 g of fresh plant yields 1 g of dry powder), corresponding to a drying yield of 20 %. The aqueous extract was prepared using the traditional Moroccan decoction method: 10 g of powdered leaves were mixed with 1 L of distilled water, boiled for 10 min and then left to cool for approximately 20 min. The resulting mixture was filtered through a Millipore membrane (0.2 µm; Millipore, Saint-Quentin-en-Yvelines, France) to remove particulate matter. Finally, the filtered extract was freeze-dried using a LABCONCO freeze-dryer (G. BOYER, Laboratory Equipment, Casablanca). The extraction yield of the lyophilised material was 20 % relative to the dry plant material.

Experimental animals

The animals used in this study were healthy young adult male albino rats (Wistar strain), weighing approximately 72–113 g, aged 8-16 weeks. They were purchased from the Missouri Experimental Centre in Morocco. The rats were housed in individual polyethene cages under standard laboratory conditions (temperature, humidity and light/dark cycle) and received a standard laboratory diet (Provimac, Morocco). This diet contained protein, lipids and fibre derived from cereals (corn, wheat and barley) and oilseed meals (soybean and sunflower), along with essential vitamins and minerals necessary for maintaining physiological health. Drinking water was available ad libitum. All applicable institution was guidelines regarding the care and use of laboratory animals were followed, in accordance with the local ethics committee of the Faculty of Science and Technology of Errachidia (approval: 2015).

Chemical reagents and drugs

We purchased indomethacin, 4-aminopyridine (4-AP), MLN-4760, EP, N-nitro-L-arginine methyl ester (L-NAME) and Methylene blue from Sigma Chemical Co. (St. Louis, USA). All other reagents were of analytical grade and obtained locally. Distilled water was used to dissolve all the drugs mentioned.

All drugs were stored under appropriate temperature and humidity conditions, as specified below:

Measurement of vascular relaxation and evaluation of the mechanisms involved

Male Wistar rats weighing between 160 and 250 g were housed in groups of five per cage under controlled conditions, with a 12 hrs light/dark cycle at 25 °C and free access to food and water. After anaesthesia with sodium pentobarbital, the animals were exsanguinated and euthanised by stunning. The thoracic aortas were immediately removed and immersed in cold buffer, then carefully dissected to eliminate adherent fat and connective tissue. In this experimental setup, isolated arterial segments were cut into rings (2 rings per animal) measuring 2–3 mm in length. These were mounted under a resting tension of 2 g in 40 mL organ baths filled with Krebs–Henseleit (KH) solution. The

Drugs	Drug storage conditions
$BaCl_2$	Store in a dry place at room temperature (15-25 °C) but prepare just before use to avoid contamination or precipitation.
Indomethacin	Stored between 2-8 °C in a dry, well-ventilated place and protected from light.
4-AP	Store at 4 °C, away from light and humidity.
MLN-4760	Stock solutions prepared in DMSO were stored at -20 °C and protected from repeated freeze thaw cycles.
EP	Stored at -20 °C, protected from light and air.
L-NAME	Aqueous solutions were stored at 2-8 °C for immediate use, or at –20 °C for short-term storage, in tightly sealed containers in a dry place.
Methylene blue	Store between 15 °C and 30 °C, in a dry place, protected from light.
Propranolol	Store between 15 °C and 30 °C, in a dry place, protected from light.
Nifedipine	Store between 2 °C and 8 °C, protected from light, as it is photosensitive.
Atropine	Store between 15 °C and 25 °C, in a tightly closed container, protected from heat and moisture.
Glibenclamide	Store in a tightly closed container away from light and humidity at a temperature between + 2 °C and + 8 °C.

solution was continuously oxygenated with a gas mixture of 95 % O_2 and 5 % CO_2 and maintained at 37 °C with a pH of 7.4 (6).

The KH solution composition (in Mm) included: NaCl (118), KCl (4.50), $NaHCO_3$ (25), glucose, NaH_2PO_4 (1.2), $MgSO_4$ (1.2) and calcium chloride ($CaCl_2$) (1.8). The rings were mounted using two stainless steel hooks one anchored to the chamber base, the other connected to a UF1 isometric force transducer (LCM Systems Ltd), which interfaced with a PowerLab/400 data acquisition system (AD Instruments, via Harvard Apparatus, Boyer, Casablanca, Morocco). The setup enabled continuous recording of isometric tension, as previously described (10). The following experiment was conducted to identify the molecular pathway underlying the relaxant effect of MMAE on (EP)-precontracted rat aortic rings, with a sample size of n=5.

The aortic rings were preincubated with one of several drug standards for 20 min before the addition of EP. The agents used for preincubation included: [1] L-NAME (10^{-4} M), which directly inhibits NO synthase; [2] Glibenclamide (10^{-5} M), an ATP-sensitive potassium channel blocker; [3] Indomethacin (10^{-5} M), a cyclooxygenase inhibitor; [4] Propranolol (10^{-5} M), a non-selective beta-adrenergic receptor antagonist; [5] Methylene blue (10^{-5} M), which interferes with the NO-Cgmp signaling pathway; [6] Nifedipine (10^{-5} M), a blocker of L-type calcium channels; [7] 4-AP (10^{-4} M), a voltage-gated potassium channel inhibitor ;[8] Barium chloride ($Bacl_2$) (10^{-4}M), an inwardly rectifying potassium channel blocker ;[9] Atropine (10^{-5} M), an inhibitor of the muscarinic action of acetylcholine; [10] MLN-4760 (6 10^{-9} M), an ACE-2 inhibitor. The contractile responses triggered by $CaCl_2$ were quantified in grams (g) and compared between preparations with and without MMAE preincubation (11).

Preliminary phytochemical screening of *M. multibracteatum*

An initial qualitative phytochemical screening of MMAE was carried out by using standard methods to detect different classes of bioactive compounds, such as polyphenols, flavonoids, tannins, saponins, quinones, anthraquinones, carbohydrates, reducing sugars, glycosides, sterols, terpenoids and alkaloids, based on established analytical procedures (12).

Determinant of total polyphenols, flavonoids and tannins content

Quantification of total phenolic compounds in MMAE was performed as per the standard protocol (13). Total flavonoid content was determined as per the standard protocol (14). Condensed tannin content was assessed using a modified version of the method as per the standard protocol (15). As for polyphenols, absorbance measurements were performed using a UV/Vis spectrophotometer at 765 nm. Gallic acid was used to generate the calibration curve. The total phenolic content was expressed as gallic acid equivalents (GAE), in mg $g^{-1}$of extract. For flavonoids, absorbance was measured at 510 nm using rutin to prepare the calibration curve. The results were expressed as rutin equivalents (RE), in milligrams per gram of extract. For tannins, absorbance was measured at 500 nm. Condensed tannins were expressed as catechin equivalents (CE), in mg $g^{-1}$of extract. All measurements were performed in triplicate (n = 3).

Evaluation of the anti-radical activity by free radical scavenging (2,2-Diphenyl-1-picrylhydrazyl: DPPH)

The antioxidant activity of MMAE was evaluated through its ability to donate hydrogen atoms or scavenge free radicals, using the stable DPPH radical. A DPPH solution (4 mg in 100 mL of methanol) was prepared and kept in the dark for 3 hr. Serial dilutions of the extract were made from a stock solution (1 mg/16 mL) to yield concentrations of 1.95 to 62.5 µg/mL. Similarly, BHT (positive control) was diluted from a 5 mg/10 mL stock solution to obtain concentrations ranging from 31.25 to 500 µg/mL.

The antioxidant activity of the MMAE was evaluated using the DPPH radical scavenging assay, following a previously described method (16). In this assay, each test tube received 5 mL of a freshly prepared DPPH solution. The mixtures were incubated in the dark for 30 min at room temperature after vortexing, while 2.5 mL of DPPH solution was mixed with 2.5 mL of methanol for the negative control. Absorbance measurements were performed using a UV/Vis spectrophotometer at 515 nm. The following equation was used to calculate the percentage inhibition of DPPH radicals:

$$I\,\% = (\text{One control} - \text{One sample} / \text{One control}) \times 100 \quad \text{(Eqn. 1)}$$

Where: I % : percentage of anti-radical activity, One control: Absorbance at 515 nm of the control (DPPH without MMAE), One sample: Absorbance of the sample containing MMAE, Concentration of the extract: expressed in µg/mL. Results were expressed as the mean ± standard error of the mean (SEM) from 3 independent experiments.

Statistical analysis

To evaluate vasorelaxant responses, a sample of n = 25 rats was used and to evaluate contractions induced by Ca^{2+} in a calcium-free Krebs solution, a sample of n = 5 rats was used. After confirming the normal distribution of the data using the Shapiro-Wilk test and the homogeneity of variances with Levenes' test, statistical analyses were conducted. A two-way ANOVA followed by Bonferronis' post-hoc test was used to assess vasorelaxant responses, while a students' t-test was applied to evaluate contractions induced by Ca^{2+} in a calcium-free Krebs solution. All statistical analyses were performed using GraphPad Prism version 8 (GraphPad Software, San Diego, CA, USA). Data are expressed as mean ± standard error of the mean (SEM) and differences were considered statistically significant at $p < 0.05$ (17).

Results

Vasorelaxant effect of MMAE on isolated aortic rings

A sample of 30 rats was studied to evaluate the vasorelaxant properties of MMAE on rat aortic rings with intact endothelium. A series of concentration-dependent experiments was carried out using 4 doses (0.75, 1, 1.25 and 1.50 mg/mL). Aortic rings were precontracted with either EP (10 µM) or KCl (80 mM). Differences were considered statistically significant when $p < 0.05$. As shown in Fig. 1, MMAE induced a significant relaxation in EP-precontracted rings starting at 0.75 mg/mL ($p < 0.05$), with maximal relaxation observed at 1.50 mg/mL ($p < 0.0001$), reaching R_{max} = 95.78 ± 2.1 % ($p < 0.0001$) (Fig. 1A). In rings contracted with KCl (Fig. 1B), a significant reduction in tension appeared from 1.25 mg/mL onward ($p < 0.001$). Control experiments using the highest volume of vehicle (distilled water) showed no appreciable vasorelaxant effect. To further

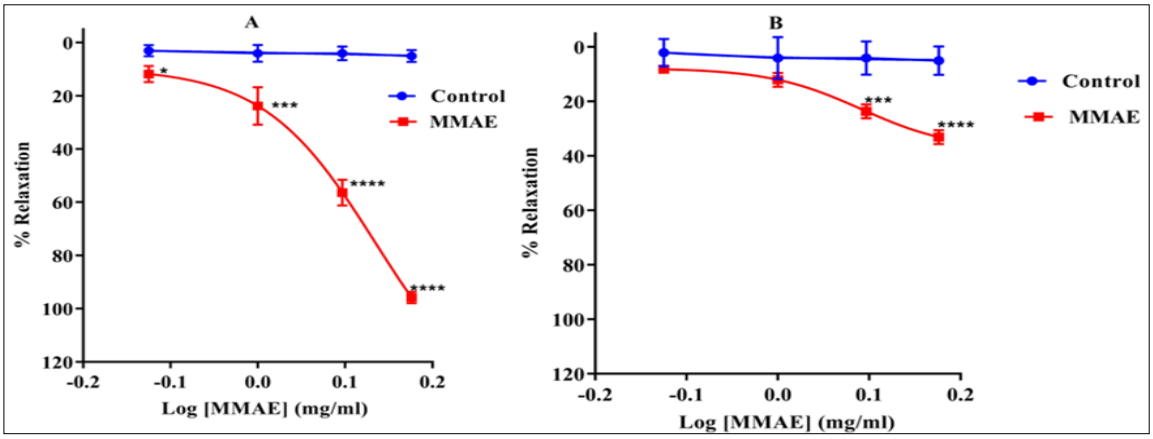

Fig. 1. Effect of MMAE (0.75, 1, 1.25 and 1.5 mg/mL) on aortic ring vasorelaxation. Concentration-dependent cumulative curves for relaxation induced by MMAE and vehicle (distilled water) in rat aortic rings. **A.** pre-contraction with EP (10 µM); **B.** pre-contraction with KCl (80 mM). Data represent the mean ± SEM; *p<0.05, ***p<0.001, ****p<0.0001.

investigate the underlying mechanisms of MMAE-induced vasodilation, additional pharmacological tools were applied after precontraction with EP (10 µM).

Effect of NO synthase inhibitor and prostaglandin synthesis Inhibitor on Aortic Responses to MMAE

To investigate the involvement of NO in the vasorelaxant response induced by MMAE, aortic rings were pretreated with L-NAME (10^{-4} M) for 20 min before contraction with EP (10 µM). As shown in Fig. 2A and Table 1, this pretreatment significantly attenuated the relaxation effect of MMAE ($p<0.05$). In contrast, without L-NAME, MMAE elicited a marked vasorelaxation from the initial dose of 0.75 mg/mL ($p<0.05$) up to 1.50 mg/mL ($p<0.0001$) (Fig. 2A). The value of R_{max} related to this effect was 65.41 ± 2.62 %. While in rings pre-incubated with L-NAME for 20 min before EP pre-contraction, the R_{max} was found to be equal to 44.02 ± 6.86 % ($p < 0.05$). In rings treated with MMAE without first being incubated with indomethacin (10^{-5} M), the R_{max} value was 90.12 ± 3.86 % (Fig. 2B). While in the rings pre-incubated with indomethacin (10^{-5} M) for 20 min before the pre-contraction of the PE (10 µM), these values were: R_{max} = 104.34 ± 3.29 % (Fig. 2B). Accordingly, pretreatment with indomethacin did not produce a significant decrease in the relaxation responses of aortic rings exposed to various doses of MMAE. Therefore, L-NAME significantly attenuated the vasorelaxant effect of MMAE, whereas indomethacin had no significant impact on the relaxation responses of aortic rings exposed to different concentrations of MMAE (Fig. 2 and Table 1).

Effect of other pharmacological inhibitors

Aortic responses to MMAE in the absence and presence of glibenclamide and nifedipine

In this experiment, rat aortic rings were pre-incubated for 20 min with Glibenclamide (10^{-5} M) and Nifedipine (10^{-5} M) before epinephrine-induced contraction (10 µM). The outcomes,

Table 1. Summary of the effects of inhibitors on aortic rings precontracted by PE. (+) means that the inhibitors significantly reduce the vaso-constrictor effect of MMAE, while (-) means the opposite

Drugs	The mechanism	Effects of inhibitors on precontracted aortic rings by EP
L-NAME (10^{-4} M)	Directly inhibits nitric oxide synthase	+
Glibenclamide (10^{-5} M)	An ATP-sensitive potassium channel blocker	-
Indomethacin (10^{-5} M)	A cyclooxygenase inhibitor	-
Propranolol (10^{-5} M)	A non-selective beta-adrenergic receptor antagonist	-
Methylene blue (10^{-5} M)	Interferes with the NO-cGMP signalling pathway	-
Nifedipine (10^{-5} M)	A blocker of L-type calcium channels	-
4-AP (10^{-4} M)	A voltage-gated potassium channel inhibitor	-
$BaCl_2$(10^{-4} M)	An inwardly rectifying potassium channel blocker	-
Atropine (10^{-5} M)	An inhibitor of the muscarinic action of acetylcholine	-
MLN-4760 (10^{-9} M)	An ACE-2 inhibitor	-

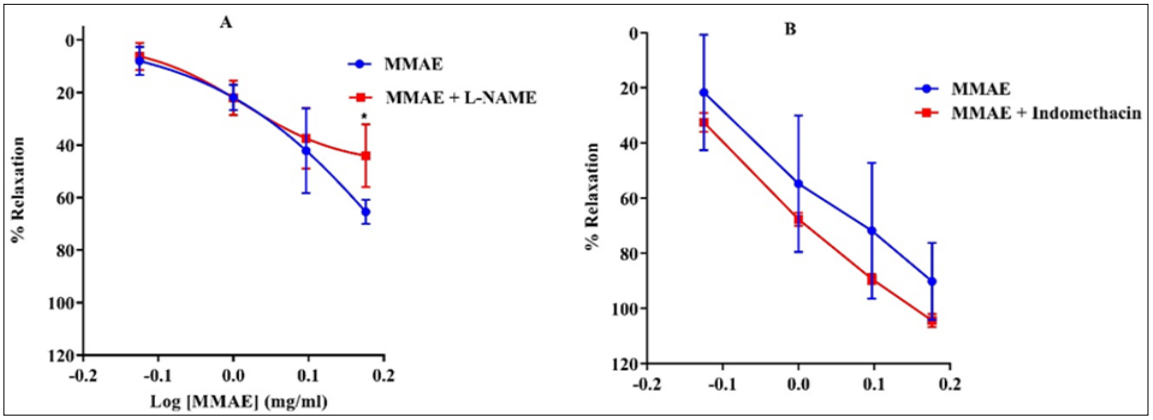

Fig. 2. Effect of different pre-incubated drugs on MMAE-induced relaxation in EP-pre-contracted aortic rings (10 µM). Aortic rings were incubated with cumulative concentrations of MMAE (0.75, 1, 1.25 and 1.5 mg/mL) in the absence and presence of pre-incubation of the following drugs: **A.** L-NAME and **B.** Indomethacin. Data represent mean ± SEM: *p<0.05.

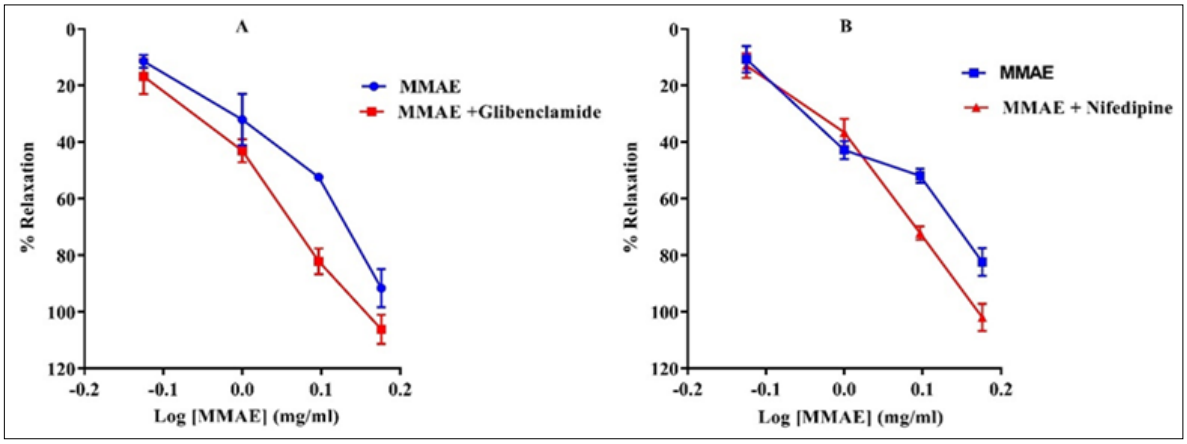

Fig. 3. Effect of different pre-incubated drugs on MMAE-induced relaxation in EP-pre-contracted aortic rings (10 µM). Aortic rings were incubated with cumulative concentrations of MMAE (0.75, 1, 1.25 and 1.5 mg/mL) in the absence or presence of pre-incubation of the following drugs: **A.** Glibenclamide, **B.** Nifedipine. Data represent mean ± SEM.

presented in Fig. 3, revealed that neither Glibenclamide nor Nifedipine significantly diminished the vasorelaxation elicited by increasing concentrations of MMAE. Notably, in the absence of Glibenclamide pretreatment, MMAE caused a significant concentration-dependent relaxation of the aortic rings, beginning at 0.75 mg/mL ($p < 0.05$) and reaching maximum effect at 1.50 mg/mL ($p < 0.0001$) (Fig. 3A). The R_{max} related to this effect was 91.61 ± 2.63 %. Whereas, in rings preincubated with Glibenclamide for 20 min before EP precontraction (10 µM), R_{max} was found to be equal to 106.15 ± 2.08 % (Fig. 3A). In rings treated with MMAE without having been previously incubated with Nifedipine (10^{-5} M), the R_{max} value was 82.38 ± 8.51 %. While in rings pre-incubated with nifedipine for 20 min before the pre-contraction of PE (10 µM), R_{max} = 102 ± 7.170 % (Fig. 3B). Therefore, Glibenclamide and Nifedipine did not significantly decrease the relaxation responses of aortic rings exposed to different doses of MMAE (Fig. 3 and Table 1).

Aortic responses to MMAE in the absence and presence of methylene blue and propranolol

In this experiment, rat aortic rings were pre-incubated for 20 min with methylene blue (10^{-5} M) and propranolol (10^{-5} M), before EP-induced contraction (10 µM). As illustrated in Fig. 4, neither methylene blue nor propranolol significantly attenuated the vasorelaxant responses to various concentrations of MMAE. In aortic rings treated with MMAE without prior incubation with methylene blue (10^{-5} M), the Rmax value was 93.2 ± 5.838 %. While in rings preincubated with BM for 20 min before EP precontraction (10 µM), the R_{max} = 95± 5.877 % (Fig. 4A). In aortic rings treated with MMAE without prior incubation with propranolol (10^{-5}M), the R_{max} value was 80.864 ± 3.125 %. While in rings pre-incubated with propranolol for 20 min before EP pre-contraction (10 µM), the R_{max} was equal to 95.94 ± 3.68 % (Fig. 4B).

Aortic responses to MMAE in the absence and presence of atropine and $BaCl_2$

Rat aortic rings were preincubated with atropine (10^{-5} M) and $BaCl_2$ in this experiment. After 20 min of preincubation before EP pre-contraction (10 µM), the outcomes were examined and depicted in Fig. 5 and Table 1. In aortic rings treated with MMAE without prior incubation with $BaCl_2$ the R_{max} value was R_{max} = 97 ± 5.838 %. While in rings pre-incubated with $BaCl_2$ for 20 min before EP pre-contraction (10 µM), the R_{max} value was equal to 97.2 ± 5.877 % (Fig. 5A). In aortic rings treated with MMAE without prior incubation with atropine (10^{-5} M), the R_{max} value was 95 ± 6.986 %. Whereas, in rings pre-incubated with atropine for 20 min before EP pre-contraction, the R_{max} was found to be equal to = 95.1 ± 5.011 % (Fig. 5B). The findings indicated that pre-incubation with atropine and $BaCl_2$ had no significant impact on the relaxation of rat aortic rings induced by increasing concentrations of MMAE (Fig. 5).

Aortic responses to MMAE in the absence and presence of 4-Aminopyridine (4-AP) and MLN-4760

The results of this experiment were analysed and shown in Fig. 6. Rat aortic rings were preincubated for 20 min with 4-AP (10^{-4} M) and with MLN-4760 (6 nM), before precontraction by EP (10 µM). These results demonstrated that pre-incubation with 4-AP, did

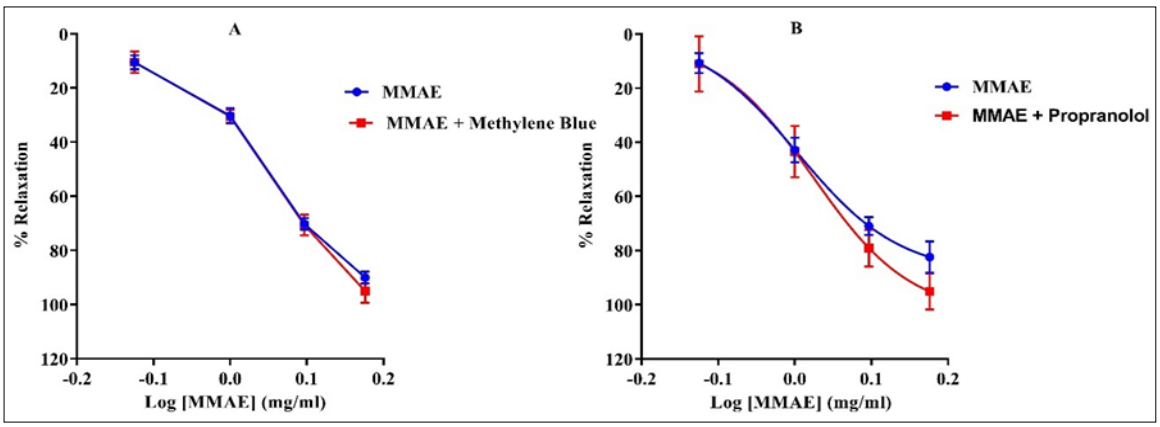

Fig. 4. Effect of different pre-incubated drugs on MMAE-induced relaxation in EP-pre-contracted aortic rings (10 µM). Aortic rings were incubated with cumulative concentrations of MMAE (0.75, 1, 1.25 and 1.5 mg/mL) in the absence or presence of pre-incubation of the following drugs: **A.** Methylene blue (MB), **B.** Propranolol. Data represent mean ± SEM.

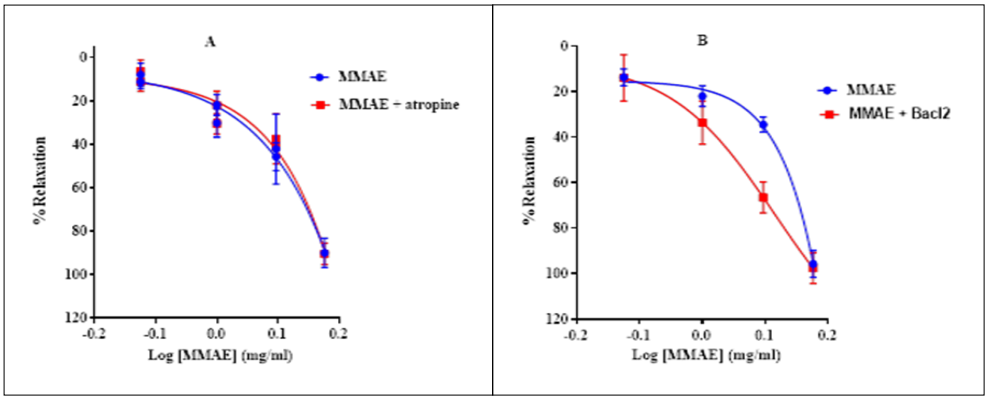

Fig. 5. Effect of different pre-incubated drugs on MMAE-induced relaxation in EP-pre-contracted aortic rings (10 µM). Aortic rings were incubated with cumulative concentrations of MMAE (0.75, 1, 1.25 and 1.5 mg/mL) in the absence or presence of pre-incubation of the following drugs: **A.** atropine, **B.** $BaCl_2$. Data represent mean ± SEM.

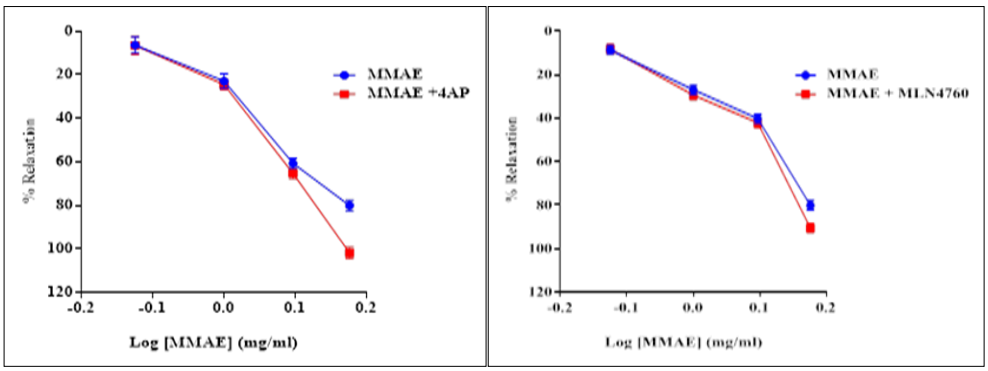

Fig. 6. Effect of different pre-incubated drugs on MMAE-induced relaxation in EP-pre-contracted aortic rings (10 µM). Aortic rings were incubated with cumulative concentrations of MMAE (0.75, 1, 1.25 and 1.5 mg/mL) in the absence and presence of pre-incubation of the following drugs: 4-AP and MLN-4760 (6 nM) (specific ACE-2 inhibitor). Data represent mean ± SEM.

not attenuate the vasorelaxant responses of rat aortic rings to cumulative concentrations of MMAE (Fig. 6A). Whereas, aortic rings treated with MMAE and precontracted by EP without 4-AP preincubation were significantly dilated by MMAE showed significant dilation from the first concentration (0.75 mg/mL, $p < 0.05$) to the last selected concentration (1.50 mg/mL, $p < 0.0001$) with a R_{max} value equal to 80 ± 5.838 % (Fig. 6A). Whereas, in the rings pre-incubated with 4-AP for 20 min before the pre-contraction of the EP (10 µM), the R_{max} value was equal to 101.72 ± 5.877 % (Fig. 6A). Furthermore, MLN-4760 had no notable effect on the relaxation of rat aortic rings in response to various concentrations of MMAE (Fig. 6B). Therefore, 4-AP and MLN-4760 did not significantly reduce the vasorelaxant responses of aortic rings exposed to various concentrations of MMAE (Table 1).

Effect of MMAE on Ca^{2+}-induced extracellular contractions

The contractile response to elevated calcium chloride ($CaCl_2$, 0.3 mM) was assessed in rat aortic rings precontracted with EP (10 µM) or KCl (80 mM) in calcium-free KH buffer, with or without preincubation with MMAE (0.75 mg/mL) for 20 min. Calcium-induced tension was recorded following the addition of $CaCl_2$ in both control and treated groups (Fig. 7). In calcium-free conditions, $CaCl_2$ triggered a gradual increase in contractile force. As depicted in Fig. 7, MMAE pretreatment significantly ($P < 0.001$) reduced the sustained contraction caused by $CaCl_2$ in EP-precontracted rings. However, in rings precontracted with KCl, MMAE failed to inhibit the prolonged contraction induced by $CaCl_2$ (0.3 mM) (Fig. 7).

Phytochemical screening and quantification

Qualitative screening of MMAE revealed the presence of polyphenols, flavonoids, tannins, saponins, alkaloids, terpenoids, glycosides, quinones and reducing sugars (Table 2).

Table 2. Phytochemical screening of aqueous extract of *M. multibracteatum.* **(+)** means the existence of the detected phytochemical group

Detected phytochemical group	Test	Results of the test
Polyphenols	Ferris chlorid test	(+)
Flavonoids	Shinoda test	(+)
Tannins	Ferric chlorid test	(+)
Saponins	Frothing test	(+)
Quinones	Sulfuric acid test	(+)
Anthraquinones	Benzene and ammonia test	(+)
Sterols and terpenoids	Libermann-Buchard test	(+)
Glycosides	Borntragers' test	(+)
Carbohydrates	Benedicts' solution	(+)
Reducing sugars	Fehlings' test	(+)
	Wagners' test	(+)
Alkaloids	Mayers' test	(+)
	Borntragers' test	(+)

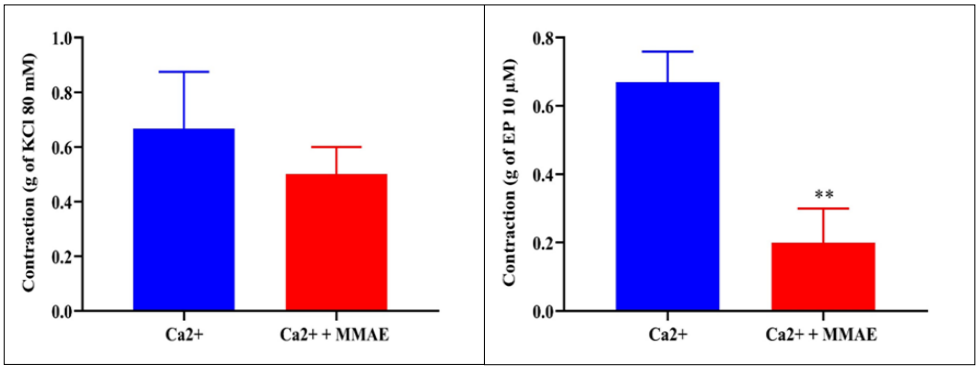

Fig. 7. Inhibitory effect of low concentration MMAE (0.75 mg/mL) on the contraction induced by the addition of cumulative concentrations of extracellular Ca^{2+} in the rat thoracic aortic rings pre-contracted by PE (10 µM) and by KCl (80 mM) in the presence and absence of MMAE. Values are expressed as mean ± SEM. ** $p<0.01$ compared to the control.

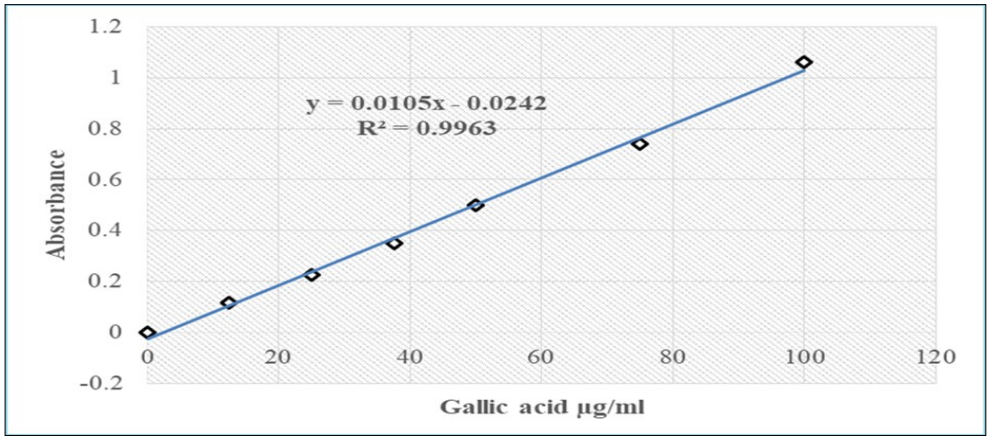

Fig. 8. Calibration curve of polyphenol contents established with gallic acid (µg/mL). absorbance measurements were performed using a UV/Vis spectrophotometer at 765 nm.

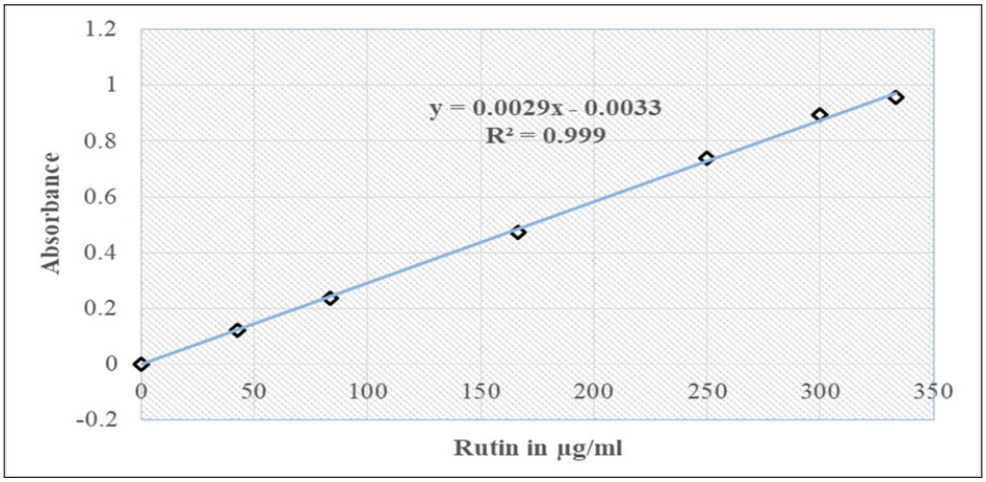

Fig. 9. Calibration curve of flavonoid contents established with Rutin (µg/mL). Absorbance was measured at 510 nm.

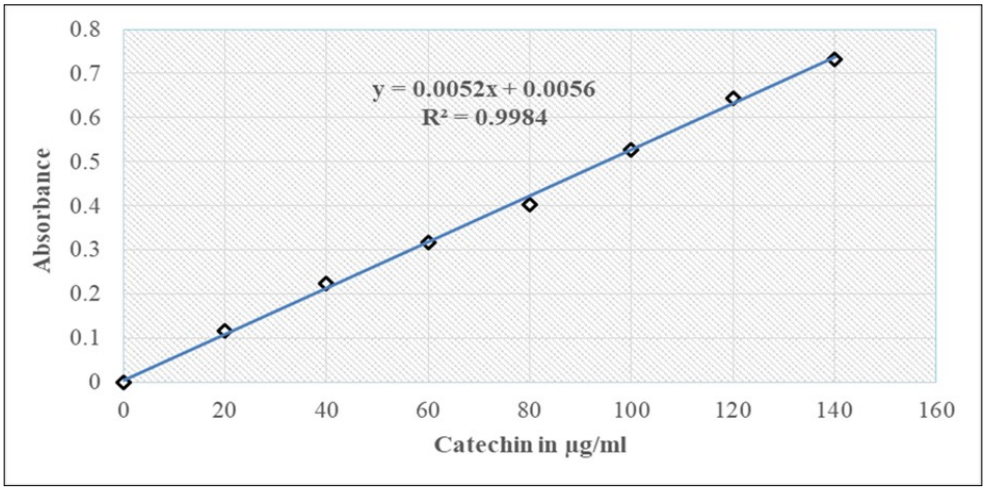

Fig. 10. Calibration curve of flavonoid contents established with Catechin (µg/mL). Absorbance was measured at 510 nm.

The quantitative content was noticed for total polyphenols 19.152 ± 2.50 mg GAE/g, Flavonoids (18.1 ± 0.57 mg RE/g) and Tannins (84.03 ± 0.432 mg CE/g) (Fig. 8-10).

Antioxidant activity

MMAE exhibited dose-dependent antioxidant activity at various concentrations (1.953; 3.9; 7.81; 15.62; 31.25; and 62.5 µg/mL), resulting in inhibition percentages of 5.59 %, 8.58 %, 11.38 %, 16.97 %, 24.06 % and 44.21 %, respectively (Fig. 11). In comparison, the standard antioxidant BHT, tested at concentrations of 31.25, 62.5, 125, 250 and 500 µg/mL, produced inhibition rates of 49.17 %, 52.89 %, 59.67 %, 68.66 % and 82.87 %, respectively (Fig. 12). IC_{50} values were determined by linear regression analysis. MMAE displayed an IC_{50} of 72 µg/mL, whereas BHT showed a significantly lower IC_{50} of 13.63 µg/mL.

Discussion

Exploration of the mechanism involved in the vasodilatory effect of MMAE using standard pharmacological agents.

To explore the mechanism involved in the antihypertensive effect observed for MMAE, an ex vivo experiment was conducted to assess its vasorelaxant properties using isolated aortic rings from Wistar rats. Thus, the results demonstrated that MMAE induced complete relaxation of aortic rings pre-contracted with EP (10 µM). Whereas it induced significant, concentration-dependent relaxation in aortic rings precontracted with KCl (80 mM). Ten standard pharmacological agents were employed in this study to help elucidate the mechanisms underlying the vasorelaxant effect of MMAE. L-NAME, a NO synthase inhibitor, indomethacin (the prostaglandin production inhibitor), Glibenclamide (an ATP-sensitive K^+channel blocker), Nifedipine (Ca^{2+} L-type calcium channel blocker), methylene blue (NO-cyclic guanosine monophosphate (cGMP) blocker), propranolol (β-adrenergic receptors blocker atropine, inhibitor of muscarinic action of acetylcholine on smooth muscle), barium chloride ($BaCl_2$), an inwardly rectifying potassium channel blocker (a KIR blocker) and with MLN-4760 (a specific ACE-2 inhibitor). On the other hand, the present study revealed that L-NAME (10^{-4} M) significantly reduced the relaxation induced by MMAE; the remaining 10 reference drugs did not alter the vasorelaxant response to MMAE.

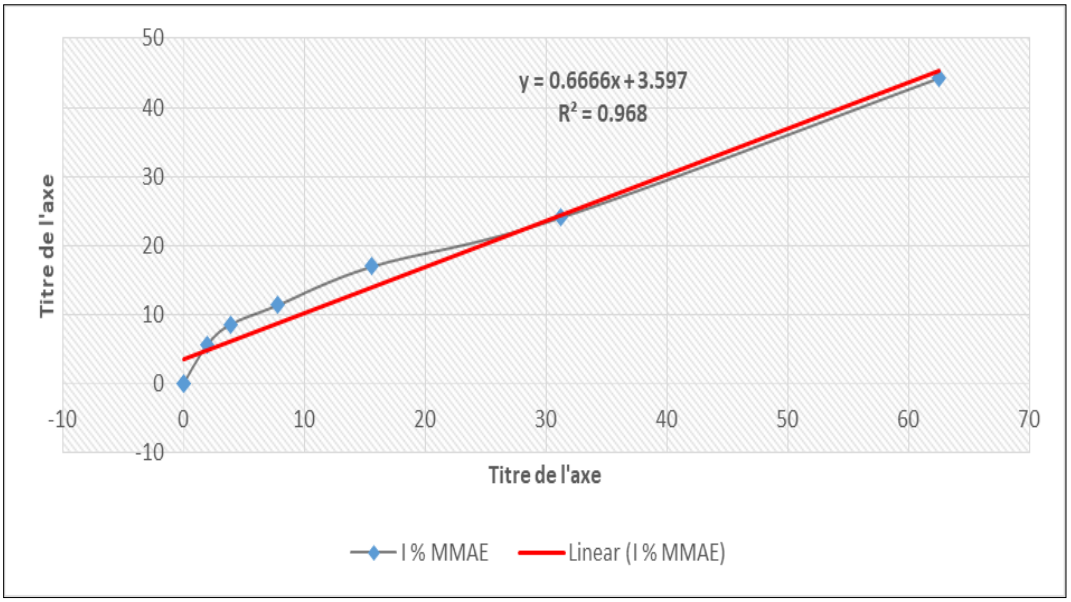

Fig. 11. Percentage of DPPH inhibition as a function of MMAE concentrations. Absorbance measurements were performed using a UV/Vis spectrophotometer at 515 nm.

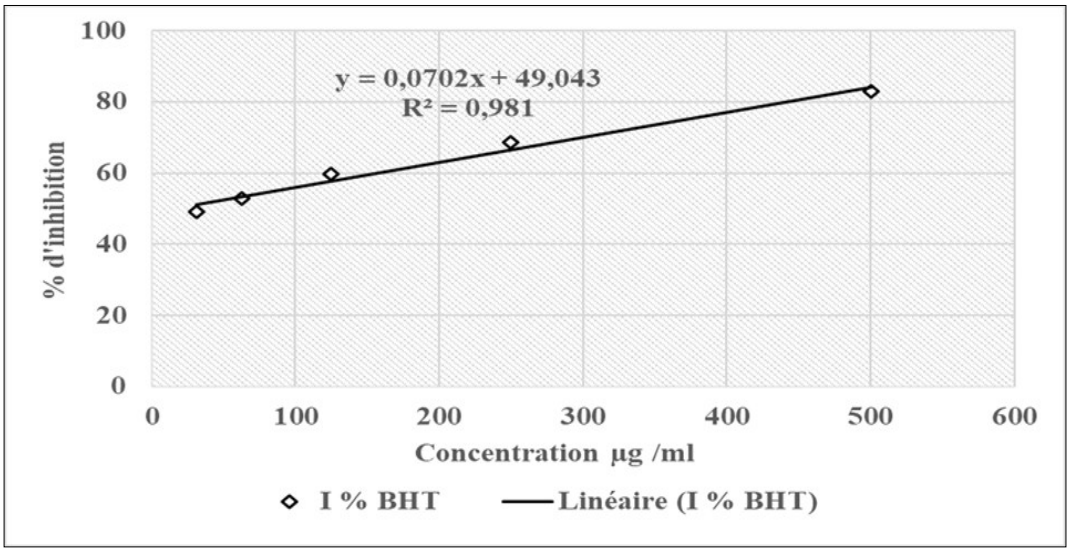

Fig. 12. Percentage of DPPH inhibition as a function of butylated hydroxytoluene (BHT) concentrations. Absorbance measurements were performed using a UV/Vis spectrophotometer at 515 nm.

Evaluating the involvement of calcium channels in the vasodilatory effect of MMAE

Furthermore, to assess the involvement of vascular smooth muscle cell calcium channels in the vasodilatory effect, $CaCl_2$-induced contraction responses were assessed in aortic rings previously contracted with EP (10 µM) or KCl (80 mM), in calcium-free KH buffer, in the presence and absence of preincubation with a low concentration of MMAE (0.75 mg/mL). Contraction of vascular smooth muscle results from an elevation of cytosolic Ca^{2+}, mediated by the activation of ROCCs and voltage-gated calcium channels (VDCCs) present in the plasma membrane. These channels can be activated by EP in the case of ROCCs and by elevated extracellular potassium levels for VDCCs, or by the release of Ca^{2+} from the sarcoplasmic reticulum (SR) (18, 19). Inhibition or suppression of these calcium channels prevents the influx or release of calcium into the cytoplasm of vascular smooth muscle cells, thus promoting relaxation and vasodilation (20), while the initial phasic contraction and transient increase in Ca^{2+} with adrenaline are thought to be due to Ca^{2+} release from the SR (21, 22). Our results demonstrated that preincubation with a low concentration of MMAE (0.75 mg/mL) for 20 min significantly inhibited only sustained contraction induced by extracellular calcium chloride ($CaCl_2$, 0.3 mM) in aortic rings precontracted with EP(10 µM).

Deduce the mechanism used for the vasorelaxant effect in the first and second experiments.

Our results showed that L-NAME significantly attenuated the vasorelaxant effect of MMAE. These suggest that the vasorelaxant effect of MMAE is mainly mediated by the direct NO pathway. Moreover, our results showed that MMAE significantly inhibited $CaCl_2$-induced contraction after PE, but had no significant effect on KCl-induced contraction, suggesting that its vasodilatory effect is mainly mediated by the inhibition of extracellular Ca^{2+} influx via the ROCC. Several studies have shown that medicinal plants and their derivatives can induce vasorelaxation by inhibiting calcium (Ca^{2+}) channels (23, 24).

Comparison between the vasorelaxant effect of *M. multibracteatum* and vasorelaxant plants.

Furthermore, studies conducted by a group of researchers showed that *in vitro* and *ex vivo*, *Marrubium vulgare* (*M. vulgare*) extract inhibited rat aortic contractile responses to norepinephrine and KCl (100 mM), but this inhibition was not affected by L-NAME (the NO synthase inhibitor) (25). While the relaxant activity of marrubenol, a diterpenoid extracted from *M. vulgare*, on rat aorta, marrubenol was found to inhibit smooth muscle contraction induced by 100 mM KCl by blocking L-type calcium channels (26), while ethanolic extracts of *M. vulgare* possess a concentration-dependent vasorelaxant effect on precontracted aortic rings with and without endothelium via a common pathway mediated by several receptor agonists, such as increasing free cytosolic Ca^{2+} levels (27). Furthermore, research has demonstrated that the aqueous extract of *M. vulgare* improved impaired endothelial function in spontaneously hypertensive rats (28). Furthermore, our study revealed that *M. multibracteatum* is rich in natural antioxidants: polyphenols, flavonoids and tannins. In the same direction, studies conducted by a group of researchers on *M. vulgare* have been shown to have additional therapeutic benefits, particularly as an antioxidant (29).

Endothelial nitric oxide synthase (eNOS) is primarily expressed in endothelial cells. It maintains central regulation of blood pressure, smooth muscle relaxation and vasodilation via peripheral nitrergic nerves and has numerous other vasoprotective and antiatherosclerotic effects (30). Furthermore, activation or increased expression of eNOS is an important mechanism for the antihypertensive effects of several pharmacological or natural compounds. Stimulation of this pathway could contribute to lowering blood pressure and restoring endothelial function (31). This vasorelaxant and antihypertensive effect is therefore linked to the chemical composition of MMAE, particularly its antioxidant molecules (polyphenols, flavonoids and tannins). According to another study, polyphenols such as quercetin, apigenin, curcumin and genistein increase the expression or activity of nitric oxide synthases (NOS), reduce oxidation by inhibiting NADPH oxidase and thus improve NO-dependent vasorelaxation (32). Additionally, according to Ismail Bouadid and colleagues, Daphne gnidium aqueous extract (DGAE) reduced blood pressure in hypertensive rats. In addition, cumulative concentrations of DGAE induced vasodilation of aortic rings isolated from rats by ROCC (23). Additionally, in a Ca^{2+}-free medium, the concentration of ligustilide (a naturally occurring compound found in some Apiaceae plants, particularly Angelica sinensis) inhibited the vasoconstriction induced by $CaCl_2$ or noradrenaline (NA). This suggests that vasodilation is linked to the release of intracellular Ca^{2+} from Ca^{2+} stores as well as the inhibition of extracellular Ca^{2+} influx via VDCC and ROCC (5). Despite this therapeutic characteristic of *M. multibracteatum*, further studies are needed to investigate the genotoxicity, nephrotoxicity and neurotoxicity of MMAE, as well as the toxicity of its bioactive compounds. Furthermore, research should aim to determine the maximum safe dose with minimal adverse effects and the minimum effective dose for *in vivo* studies.

Conclusion

This study, considered the first on *M. multibracteatum*, shows that MMAE induces significant vasorelaxation of aortic rings precontracted by PE and KCl, via activation of the NO pathway and blockade of ROCC, thus demonstrating the antihypertensive potential of MMAE. This effect is related to its chemical composition and antioxidant activity. Further phytochemical and preclinical studies are needed to isolate the active compounds, as they contain compounds that, once purified, allow the discovery of antihypertensive and antioxidant drugs. Despite this therapeutic characteristic of MMAE, toxicological evaluations are also necessary, especially for long-term use.

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Authors' contributions

SE conducted vasorelaxation and phytochemical studies and wrote the manuscript. OF, AQ and IB participated in the vasorelaxation activity of the study and participated in writing the manuscript. AA[1], AA[2], MA, MH and AH participated in the study design and performed the statistical analysis. NL and FK participated in the phytochemical activity of the study. ME conceived the study and participated in its design and coordination. All authors read and approved the final manuscript [AA[1] - Ayoub Amssayef and AA[2] - Amine Azzane].

Compliance with ethical standards

Conflict of interest: The authors declare no conflict of interest, financial or otherwise.

Ethical issues: All applicable institutional guidelines for the care and use of animals were followed according to the local committee, Faculty of Sciences & Techniques Errachidia/2015.

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