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Endothelium-dependent vasorelaxation effects of F5 fraction of *Crinum amabile* chloroform extract

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Abstract

Background Vascular dysfunction can lead to many health problems including hypertension and heart disease. The complexities of vascular dysfunction and vascular disorder-related diseases have prompted the search for many new biologically active compounds in the efforts of resolving the problems. Previous studies have shown that *Amaryllidaceae* alkaloids exert multiple biological activities, including the vasorelaxation effect. *Crinum amabile,* which is a family member of *Amaryllidaceae*, is believed to possess a promising pharmacological activity as a vasorelaxant.

Method The vasorelaxation activities of *Crinum amabile* extracts and fractions were determined using in vitro models of phenylephrine pre-contracted intact and denuded rat aortic rings. The mechanistic pathways of vasorelaxation were investigated by pre-treatment of endothelium-intact rat aortic rings with L-NG Nitro Arginine Methyl Ester (L-NAME), methylene blue (MB), indomethacin, atropine and propranolol, respectively.

Results The results showed that chloroform extract (CE) of *Crinum amabile* exhibited the highest vasorelaxation activity, and further fractionation of CE found that its F5 fraction exerted the strongest activity. An in-depth study on the mechanistic pathway revealed that the endothelium-dependent vasorelaxation induced by F5 fraction was primarily achieved through stimulation of prostaglandin (PGI_2) production and partially associated with NO/cGMP activation pathway.

Conclusion Findings from this study suggest that *Crinum amabile* can serve as a promising candidate for the discovery and development of the new vasorelaxant drug.

Keywords *Crinum amabile*, F5 fraction, Vasorelaxation, Prostaglandin (PGI_2), NO/cGMP activation pathway

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1 Background

For thousands of years ago, medicinal plants have been traditionally used across the world in remedying various ailments [1]. In fact, according to a WHO report, about 80% of the world's total population depends on herbal medicines for their primary health care need due to lack of advanced medical services and financial constraints [2]. For this reason, traditional medicine has become an integral part of health system in developing countries [3].

One of the causes of vascular dysfunction is the perturbation of the endothelial lining of the vascular system. This leads to disturbances in the release of endothelial

factors such as prostacyclin (PGI2), nitric oxide (NO), and endothelium-derived hyperpolarization factor (EDHF), and subsequently the development of disorders such as hypertension, heart failure, peripheral arterial diseases and many other vascular-related diseases [4]. The mechanism of the endothelium mediated vasodilation process involves the action of endothelium-derived relaxing factor such as the nitric oxide (NO) and prostacyclin [5]. These are strong vasodilators that are released by the vascular endothelial cells as response to stress signals [6]. While the vasodilatory effects of NO occur via the cGMP pathway and activation of the enzyme protein kinase, resulting in a decrease in intracellular Ca^{2+} concentration [5, 7], prostacyclin 2 stimulates the production of cAMP, which in turn activates the protein kinase enzyme and lowers intracellular Ca2 + concentration. Plant-derived bioactive compounds exhibit vasodilatory effects by stimulating endothelial nitric oxide synthase activity, which regulates the activity of ion channels [5].

Many medicinal plants have been reported to exhibit vasorelaxation effects [8]. More than 200 plant-derived bioactive compounds have been evaluated for their vasodilation effects [9]. Amaryllidaceae alkaloids were among to be identified to exert vasorelaxation activity. Apart from that, Amaryllidaceae alkaloids were also documented to possess other pharmacological effects including antitumor, antidepressant and anti-inflammatory activities [10, 11].

Crinum amabile, as a family member of Amaryllidaceae [12], is believed to be vasoactive. A few previous studies have demonstrated the vasorelaxation effects of alkaloids isolated from *Crinum amabile* [11, 13]. Lycorine is the most commonly found alkaloid within the Amaryllidaceae family [14] while others include Maybelline, sardine and Hazeltine, which were also shown to exhibit antihypertensive activity [11]. So far, no previous study has described the vasoactivity of *Crinum amabile* leaf extract and the underlying mechanistic pathway(s) involved. Therefore, this study was conducted to investigate the vasoactive property of *Crinum amabile* leaves extracts and fractions on phenylephrine pre-contracted rat thoracic aortas, as well as to elucidate the possible mechanistic pathway(s) involved.

2 Methods

2.1 Collection of plant sample

Fresh leaves of *Crinum amabile* were collected from the vicinity of Universiti Sains Malaysia (USM). They were cleaned, cut into pieces and dried in the oven at 50 °C for 4–5 days [15]. The dried leaves were subsequently grounded into powder using an electric grinder and kept at 4 °C prior to the extraction procedure [15]. A voucher specimen was deposited at the Biodiversity Unit, Institute of Bioscience, Universiti Putra Malaysia (UPM), under the number SK 3365/18.

2.2 Experimental animals

Healthy male Sprague–Dawley rats aged between 8–10 weeks and weighing approximately 180–220 g were utilized in the study. They were kept in a ventilated room, regulated at 24 ± 44 °C temperature, 12/12 h light/dark cycle and were allowed free access to food and filtered water. All animals were acclimatized to laboratory conditions at least 7 days prior to experiments. All experimental protocols were conducted in accordance with the rules and regulations set by the Institutional Animal Care and Use Committee of UPM under AUP No: UPM/IACUC/AUP-R048/2017.

2.3 Serial extraction of *Crinum amabile* leaf powder using soxhlet' apparatus

Petroleum ether extract (PE), chloroform extract (CE), methanol extract (ME) and water extract (WE) of *Crinum amabile* leaf powder were prepared through serial extraction according to the previous method described [15]. All extracts were filtered and concentrated with a rotary evaporator before being frozen for 2 days at −80 °C and evaporated to dryness for another 2 days using a freeze-dryer. Dried extracts were stored in respective airtight containers, in the desiccator [16] to prevent microbial growth and deterioration caused by moisture [17]. Stock solutions of 1 g/mL of extracts in 100% dimethyl sulfoxide (DMSO) were prepared and kept at − 20 °C prior to use.

2.4 Fractionation of *Crinum amabile* chloroform extract using column chromatography

The fractionation of CE was performed in accordance with the previously established method [15]. Ten grams of CE and 20 g of silica gel were dissolved together in chloroform after which the mixture was evaporated to dryness and powdery using a rotor-evaporator. A packed silica column was then set up with the powdered CE placed on top of the column and covered with a layer of cotton wool. Subsequently, the column was eluted with a series of petroleum ether: chloroform: methanol solvent mixtures (200 mL) as described in Table 1 [15]. Each respective solvent mixture was allowed to completely auto-elute before the subsequent solvent mixture was added. Each successive eluate was collected separately into respective conical flasks and concentrated using a rotor-evaporator.

Table 1 The ratios used in the preparation of different solvent mixtures for the fractionation of *Crinum amabile* leaf chloroform extract (CE)

	Percentage (%)										
Petroleum ether	100	80	60	40	20	0	0	0	0	0	0
Chloroform	0	20	40	60	80	100	80	60	40	20	0
Methanol	0	0	0	0	0	0	20	40	60	80	100
Fraction	Wax	F1	F1	F2	F2	F3	F3	F4	F4	F5	F6

Each solvent ratio was prepared in triplicates with a volume of 200 mL each

2.5 Thin layer chromatography (TLC) profiling of CE fractions

Thin Layer Chromatography (TLC) profiling was conducted on all the CE fractions [18], using 5 × 10 cm TLC aluminium plates. A 1 cm line was first drawn from the bottom edge of the plate. Each concentrated eluate was then spotted onto the line using a fine capillary tube and left to air dry. Various mobile phases (petroleum ether: ethyl acetate (4:1, 3:2, 1:1 and 1:2), ethyl acetate: methanol (1:1), and chloroform: methanol (3:2, 1:1 and 2:3)) were prepared. Each respective mobile phase was poured into a closed TLC chamber and left to equilibrate at room temperature for 10 min. The spotted TLC plate was then placed vertically, one at a time into the solvent vapour saturated chamber using forceps and left to develop for approximately 25 min. The process was stopped, immediately once the mobile solvent reached the top edge of the plate, by removing the developed TLC plate from the chamber and then left to air dry. The procedures were repeated for each eluate using different mobile phases. All the developed TLC plates were visualized under long wavelength (365 nm) and short wavelength (254 nm) using UV transilluminator. The Rf value for each separated spot on each respective TLC plate was calculated using the following formula:

$$\text{Rf} = \text{distance travelled by the spot} / \text{distance travelled by the mobile phase}$$

Eluates with more than 85% similarities in TLC profile were pooled together into one fraction. Stock solution of 200 mg/mL in 100% DMSO for each respective fraction was prepared and stored as previously suggested in the literature [16, 17].

2.6 Gas chromatography–mass spectrometry analysis of F5 fraction of *Crinum amabile* chloroform extract

The F5 fraction of the *Crinum amabile* chloroform extract was further analysed by GC–MS using Agilent 19091S-433 system (USA). The F5 fraction was run for GC at a concentration of 10 mg/ml, 4 mg/ml and 2 mg/ml designated as F10, F4 and F2 respectively. Analysis of the F5 fraction was achieved using the following GC–MS conditions; Capillary column with nominal length of 30 m, nominal diameter of 250 µm and nominal thickness of 0.25 µm. Gas Type: Helium, Initial flow: 1.2 mL/min Sample Pumps 6, Injection Volume 1.0 µL. The oven conditions employed were; Initial temp: 70 °C (On), Maximum temp: 325 °C, Initial time: 2.00 min, Equilibration time: 0.50 min with a run time of 32.75 min. Finally, the MS scan condition used were; Solvent Delay: 3.00 min, Resulting EM Voltage: 1905.9, Threshold: 150. Mass scan range 35–650. MSZones; MS Quad: 150C maximum 200 C, MS Source: 230 °C maximum 250 °C. Compounds present in fraction F5 were identified by the comparing retention times, peak areas, peak heights, and mass spectra patterns with known compounds available in the National Institute of Standards and Technology (NIST) library.

2.7 Vasorelaxation effects of *Crinum amabile* leaves extracts and fractions

Phenylephrine (100 µM), acetylcholine (100 µM), L-NG Nitro Arginine Methyl Ester (L-NAME) (1 mM), methylene blue (MB) (1 mM), propranolol (100 µM) and atropine (100 µM) were prepared in distilled water [19] while indomethacin (1 mM) was dissolved in DMSO. All stock solutions were kept at − 20 °C prior to use unless otherwise stated.

The vasorelaxation activities of all the extracts and fractions were determined using an *in-vitro* rat aortic ring assay. The rat was subjected to general anesthesia by inhalation of carbon dioxide. The thoracic part was dissected. The thoracic aorta between the aortic arch and diaphragm was quickly isolated and transferred into a petri dish filled with Krebs' solution, and constantly bubbled with carbogen (95% O_2 and 5% CO_2). All the surrounding connective tissues and adhering fats were gently removed from the aorta after which it was cut into 3–5 mm length aortic rings. Each aortic ring was carefully hung horizontally into respective tissue chamber, through the lumen of the aortic ring, between an L-shaped stick and a haggle, which was connected to a force transducer of PowerLab equipment. Each respective tissue chamber contained 10 mL of Krebs' solution with continuous supply of

carbogen and temperature maintained at 37 °C. The aortic rings were then allowed to equilibrate under 1 g resting tension for 30 min [20, 21]. The endothelium integrity of each aortic ring was determined by pre-contraction with phenylephrine (1 μM) until a maximum contraction was attained, and then followed by relaxation with acetylcholine (1 μM). The percentage of contraction by phenylephrine was calculated with respect to its initial value before contraction, while the percentage of relaxation by acetylcholine was quantified based on the value of phenylephrine-induced contraction [22]. Aortic rings that produced > 60% contraction in response to phenylephrine and > 50% relaxation in response to acetylcholine were considered as endothelium intact. After endothelial integrity of the aortic rings were determined, they were rinsed twice with Krebs' solution and the tension was returned to baseline before proceeding with the next experiments.

All the extracts and fractions were tested on the endothelium-intact aortic rings for their vasoactive property. After the establishment of endothelium integrity, the aortic rings were again contracted with phenylephrine until a maximum plateau was achieved. One hundred microliter (100 μl) of test samples either the extracts, fractions, or negative control (0.1% DMSO), were added cumulatively into the respective organ bath at a concentration range of 0.16–10.00 mg/mL. Each concentration was added at 30-min intervals before the next dose [21, 23, 24]. The vasorelaxation effect of each test sample, at each respective cumulative concentration was recorded using PowerLab equipment and the percentage of relaxation of each test sample at each respective cumulative concentration was calculated. A dose response curve of percentage relaxation against cumulative concentration for each test sample was then plotted, in which each EC_{50} (concentration that produced 50% relaxation relative to maximum contraction) was calculated.

2.8 Endothelium-dependent vasorelaxation of *Crinum amabile* leaves chloroform extract and its F5 fraction

The above procedures were repeated by using endothelium-denuded aortic rings to evaluate the involvement of endothelium-dependency in the vasorelaxation effects of the test samples. Endothelium-denuded aortic rings refer to those rings with no observable relaxation effect or produced lower than 10% of relaxation in response to acetylcholine [25]. This can be achieved by gently rubbing the internal cells (lumen) of the aorta rings with a moistened cotton swab to mechanically remove their endothelium [26]. After that, the dose–response curves of the endothelium-denuded and endothelium-intact of each test sample were compared. The calculated EC50 between these two sets of experiments were also compared.

2.9 Investigation on the possible mechanism(s) of F5-induced endothelium-dependent vasorelaxation

In this study, the possible involvement of nitric oxide (NO), cyclic guanosine monophosphate (cGMP) or cyclooxygenase (COX) in the endothelium-dependent vasorelaxation activity of F5 fraction was investigated. The endothelium-intact aortic ring was pre-incubated with either 10 μM of L-NAME (nonspecific nitric oxide synthase inhibitor), 10 μM of MB (a cGMP inhibitor), or 10 μM of indomethacin (non-selective COX inhibitor) for 20 min prior to contraction with phenylephrine [24, 27, 28]. In addition, the possible agonistic effects of F5 fraction on muscarinic and β-adrenergic receptors were also assessed by pre-incubating the endothelium-intact aortic rings with 1 μM atropine (a competitive and non-selective muscarinic receptor antagonist) or 1 μM propranolol (a non-selective β-blocker), respectively for 20 min prior to contraction with phenylephrine [20, 24]. A total of 8 aortic rings were used for each test sample in every experimental protocol.

2.10 Statistical analyses

All data were expressed as Mean ± Standard Error Mean (SEM). Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by a post hoc Dunnett's test using the Statistical Package for Social Science Version 22 software (SPSS Inc., Chicago, IL). A p value of less than 0.05 ($p < 0.05$) was considered significant.

3 Results

3.1 Bioactive compounds identified via GC–MS analysis of F5 fraction of *Crinum amabile* chloroform extract

The GC–MS analysis of the F5 fraction of *Crinum amabile* Chloroform Extract showed the presence of 24, 12 and 11 peaks at a concentration of 2 mg/ml, 4 mg/ml and 10 mg/ml of the F5 Fraction of *Crinum amabile* Chloroform extract (Figs. 1, 2, 3). Analysis of the peaks and comparison with the National Institute of Standards and Technology (NIST) data base reveals the presence of about 45 compounds. The major compounds identified include; Heptacosane, Benzenamine, Estradiol, 3,4-Dimethyl-2-phenyltetrahydro-1,4-thiazine, 3-Chloro-N-(9,10-dioxo-9,10-dihydroantracen-1-yl)-propionamide, 3-Amino-5-(3-indolyl)-4-pyrazolecarbonitrile Galantamin, and Buphanisine 2, 1,2-Benzenedicarboxylic acid (Tables 2, 3, 4).

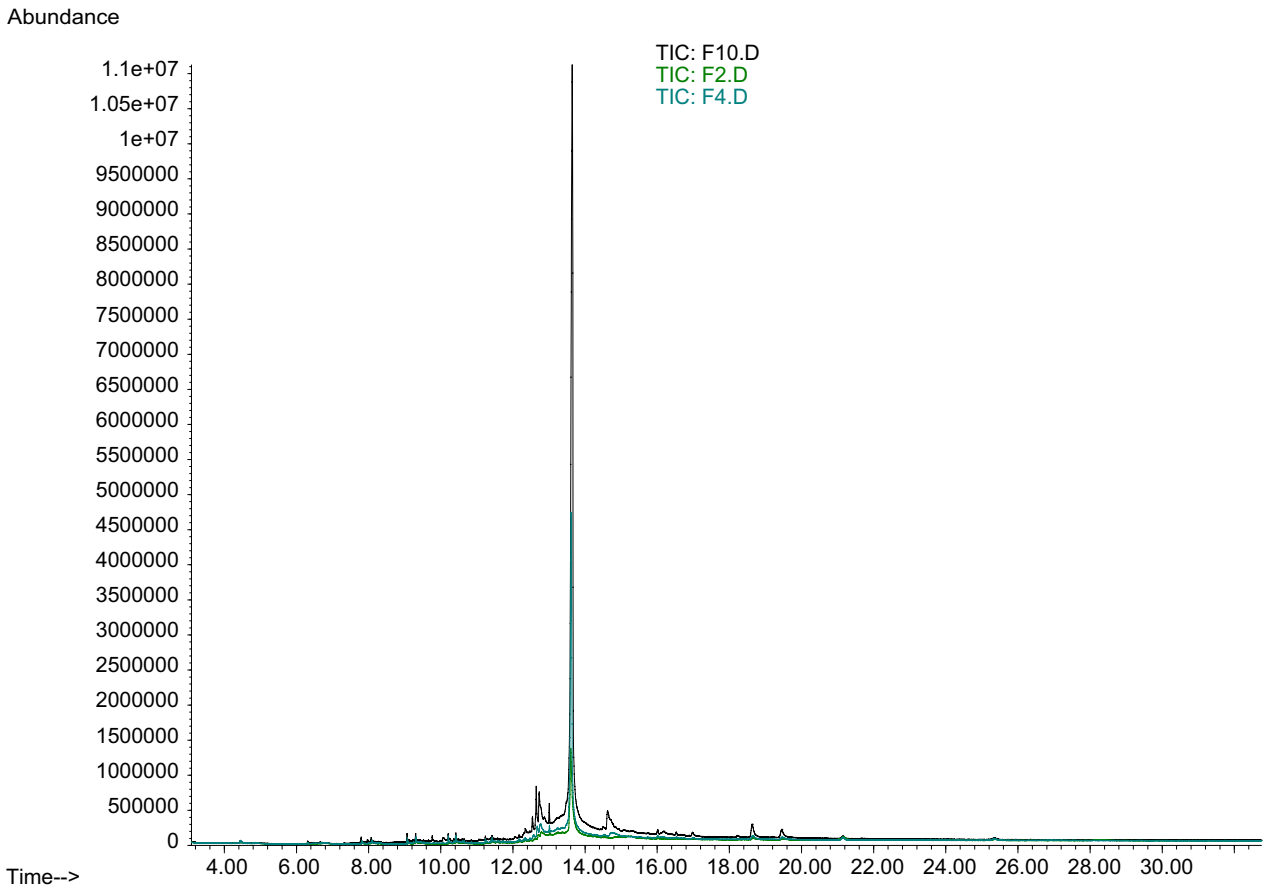

Fig. 1 Chromatogram obtained from GC–MS analysis of the F5 fraction of chloroform extract of *Crinum amabile*. F10 represents the fraction F5 running the GC at a concentration of 10 mg/ml, F2 represents the fraction F5 running the GC at a concentration of 2 mg/ml and F4 represents the fraction F5 running the GC at a concentration of 4 mg/ml

3.2 Vasorelaxation effects of *Crinum amabile* leaves extracts and fractions

Tables 5 and 6 summarized the vasorelaxation effects of *Crinum amabile* leaves extracts and fractions. All responses were expressed as EC50 and the maximum percentages of relaxation (R_{max}) values. All R_{max} values were recorded at the highest cumulative concentration tested in the respective organ bath. From the results, all the extracts exhibited a concentration-dependent vasorelaxation effect towards phenylephrine pre-contracted endothelium-intact rat aortic rings (Fig. 4). The EC50 and R_{max} values obtained were 0.67 mg/mL (R_{max}: 92.65 ± 4.52%), 0.22 mg/mL (R_{max}: 94.72 ± 6.04%), 1.41 mg/mL (R_{max}: 66.92 ± 8.16%) and 3.48 mg/mL (R_{max}: 65.37 ± 7.71%) for PE, CE, ME and WE, respectively (Table 5). Only PE and CE showed statistically significant vasorelaxation effects at all the cumulative concentrations tested, except the lowest cumulative concentration (< 0.16 mg/mL). The vasorelaxation effect of ME was only significant at the highest cumulative concentration tested (9.84 mg/mL). WE, on the other hand, showed a vasorelaxation effect comparable to that of negative control and hence, was considered to exhibit no vasorelaxation effect.

All the six fractions were also found to induce vasorelaxation effects against phenylephrine pre-contracted endothelium-intact rat aortic rings in a concentration-dependent manner (Fig. 5). The EC50 values recorded with F1 to F6 fractions were: 106.37 µg/mL, 94.76 µg/mL, 91.12 µg/mL, 140.24 µg/mL, 81.73 µg/mL and 499.53 µg/mL respectively, while the R_{max} values calculated were 96.04 ± 7.05%, 87.89 ± 4.75%, 82.12 ± 4.27%, 85.63 ± 2.83%, 100.07 ± 4.28% and 45.74 ± 4.06% respectively (Table 6). Only F2 and F5 fractions produced significant concentration-dependent vasorelaxation effects at a cumulative concentration above 109.38 µg/mL, with the F5 fraction exhibiting the strongest vasorelaxation activity, coupled with the highest R_{max} value and significance level compared to the other fractions at the same cumulative concentrations tested. F1, F3 and F4 fractions on the other hand showed significant effects only at cumulative

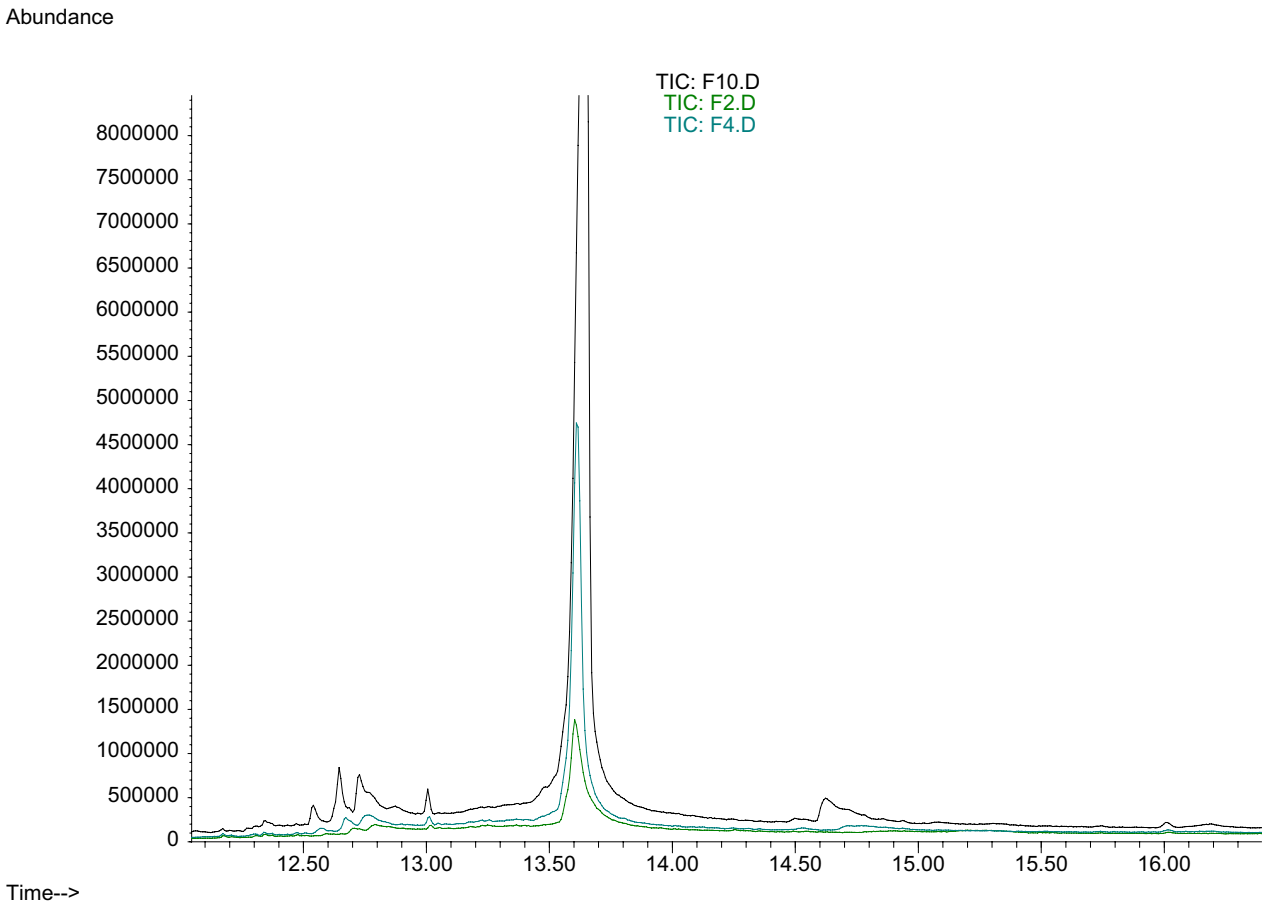

Fig. 2 Chromatogram obtained from GC–MS analysis of the F5 fraction of chloroform extract of *Crinum amabile*. F10 represents the fraction F5 running the GC at a concentration of 10 mg/ml, F2 represents the fraction F5 running the GC at a concentration of 2 mg/ml and F4 represents the fraction F5 running the GC at a concentration of 4 mg/ml

concentrations above 234.38 µg/mL, whereas the F6 fraction was considered to have no vasorelaxation activity since the value recorded was lower than that of the negative control. The vasorelaxation activities of all the fractions can be arranged in decreasing order as follows: F5 > F2 > F1 > F3 > F4.

3.3 Endothelium-dependent vasorelaxation of *Crinum amabile* leaves chloroform extract and its F5 fraction

The data showed that the vasorelaxation effect caused by CE on endothelium-denuded rat aortic rings was slightly lesser than that of endothelium-intact rat aortic rings (Fig. 6). Only a small increase in EC50 value was recorded, i.e., from 2.74 mg/mL in endothelium-intact rat aortic rings to 3.97 mg/mL in endothelium-denuded rat aortic rings. However, their R_{max} values determined at a cumulative concentration of 4.84 mg/mL were comparable with one another with 59.84 ± 5.63% and 56.34 ± 3.19%, for endothelium-intact and endothelium-denuded rat aortic rings, respectively. However, the changes (small down-shift and the R_{max} value) observed in endothelium-denuded rat aortic rings were insignificant at all the cumulative concentrations tested compared to endothelium-intact rat aortic rings.

In F5-induced vasorelaxation, there was a marked decline in vasorelaxation activity starting from a cumulative concentration of 46.88 µg/mL until the final cumulative concentration of 484.38 µg/mL in the endothelium-denuded rat aortic rings compared to endothelium-intact rat aortic rings (Fig. 7). F5-treated endothelium-denuded rat aortic rings showed a significant increase in the EC50 value, from 77.60 µg/mL in endothelium-intact rat aorta rings to 300.11 µg/mL in endothelium-denuded rat aortic rings (Table 7). The R_{max} values calculated for both endothelium-intact and endothelium-denuded rat aortic rings were 86.11 ± 2.14% and 70.11 ± 4.00% respectively which were determined at a cumulative concentration of 484.38 µg/mL. There was an approximately 16% reduction in the vasorelaxation effect.

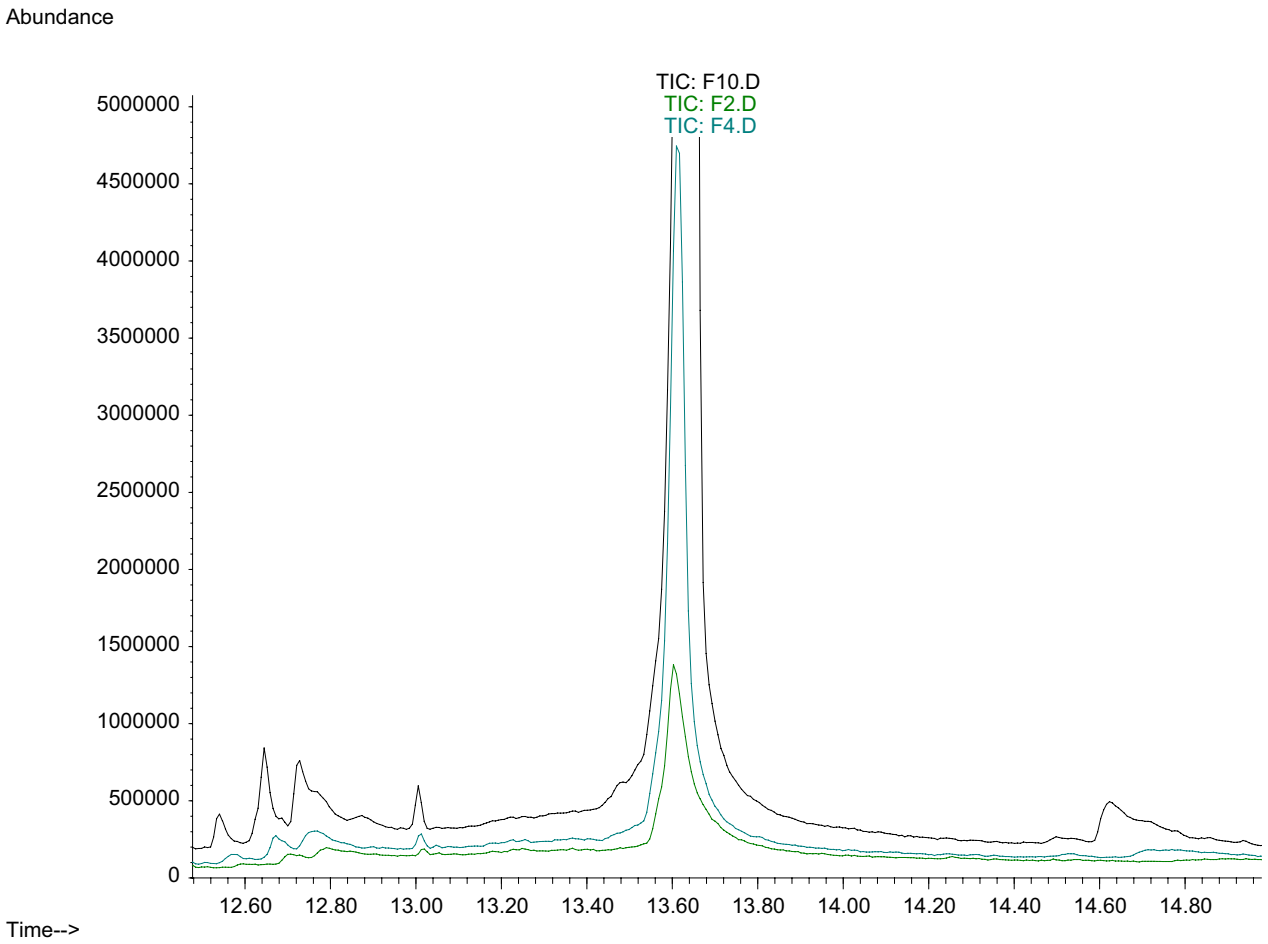

Fig. 3 Chromatogram obtained from GC–MS analysis of the F5 fraction of chloroform extract of *Crinum amabile*. F10 represents the fraction F5 running the GC at a concentration of 10 mg/ml, F2 represents the fraction F5 running the GC at a concentration of 2 mg/ml and F4 represents the fraction F5 running the GC at a concentration of 4 mg/ml

3.4 Possible mechanism(s) of F5-induced endothelium-dependent vasorelaxation

Respective EC_{50} values, R_{max} values, and p values of F5-induced endothelium-dependent vasorelaxation in the presence of receptor blockers or antagonists are summarized in Table 7. Among all the antagonists and blockers used, indomethacin was found to suppress the concentration-dependent vasorelaxation caused by F5 fraction, at all the cumulative concentrations tested except at a low cumulative concentration of 15.63 µg/mL (Fig. 8). A total of 28% reduction in the R_{max} value was observed as compared to its normal F5 fraction treatment, at the same cumulative concentration (Table 4). The EC_{50} value also increased significantly to 394.32 µg/mL, which is an approximately 400% increment compared to its normal counterpart. Meanwhile, the vasorelaxation effect of the F5 fraction in the presence of L-NAME was found to be attenuated (Fig. 8), in which a large increase to 231.17 µg/mL of EC_{50} value was recorded. In addition, there was a mild reduction to 73.47 ± 4.74% in the R_{max} value which eventually reached a negligible level compared to the normal F5-treated group. Methylene blue pre-incubated endothelium intact rat aortic rings did not significantly modify the R_{max} value (87.78 ± 4.87%) but increase the EC_{50} value to 206.76 µg/mL. On the other hand, the vasorelaxation effect exerted by the F5 fraction was not altered by the presence of atropine or propranolol (Fig. 8). The EC_{50} and R_{max} value calculated in the presence of atropine was 96.72 µg/mL and 85.83 ± 4.75%, respectively, while the presence of propranolol recorded an EC_{50} and R_{max} value of 83.13 µg/mL and 75.18 ± 3.69% respectively. The vasorelaxation response of F5 fraction in the presence of receptor blockers and antagonists can be arranged in the following order: indomethacin > MB > L-NAME while atropine and propranolol showed no influence on F5-induced vasorelaxation.

Table 2 Bioactive compounds identified in the F5 fraction (2 mg/ml) of *C. amabile* by GC–MS analysis

Peak	Retention time(Rt)	Area %	Identified compounds
1	4.45	0.36	Trichloromethane
2	7.83	0.67	Tetracosane
3	8.10	0.43	Hexadecane
4	9.08	0.71	Pentacosane
5	9.32	0.56	Octacosane
6	10.21	0.62	Heptacosane
7	10.42	0.80	Heptacosane
8	11.24	0.45	Octadecane
9	11.43	0.89	Heptadecane
10	12.18	0.33	Eicosane
11	12.35	0.84	Docosane
12	12.48	0.39	3,4-Dimethyl-2-phenyltetrahydro-1,4-thiazine
13	12.60	0.79	Ethofumesate
14	12.71	2.68	9H-Carbazole-2-ethanamine
15	12.80	8.92	3-Chloro-N-(9,10-dioxo-9,10-dihydroantracen-1-yl)-propionamide
16	13.02	3.17	Bis(2-ethylhexyl) phthalate
17	13.10	1.25	Benzenamine
18	13.19	2.89	9H-Carbazole-2-ethanamine
19	13.26	4.18	3-Amino-5-(3-indolyl)-4-pyrazolecarbonitrile
20	13.37	3.02	Benzenamine
21	13.40	1.69	Benzenamine
22	13.61	59.26	Estradiol
23	18.66	1.69	Tetrasiloxane
24	21.16	3.39	Cyclotrisiloxane

Table 3 Bioactive compounds identified in the F5 fraction (4 mg/ml) of *C. amabile* by GC–MS analysis

Peak	Retention time (Rt)	Area %	Identified compounds
1	9.08	0.49	Heneicosane
2	9.31	0.50	Pentacosane
3	10.21	0.25	Hexadecane
4	10.42	0.68	Heptacosane
5	11.42	0.42	Heptadecane
6	12.58	1.03	Galantamin
7	12.67	2.58	Buphanisine
8	12.77	6.33	Pyrimidine-5-carbonitrile
9	12.90	1.86	6-(Methylamino)phenanthren-3-ol
10	13.01	2.16	1,2-Benzenedicarboxylic acid
11	13.23	3.45	9H-Carbazole-2-ethanamine
12	13.62	80.25	Estradiol

Table 4 Bioactive compounds identified in the F5 fraction (10 mg/ml) of *C. amabile* by GC–MS analysis

Peak	Retention time (Rt)	Area %	Identified compounds
1	9.07	0.20	Undecane
2	12.35	1.31	1H-Indene-1,3(2H)-dione, 2-(2-pyridinyl)
3	12.54	1.71	Galantamin
4	12.65	2.77	Buphanisine
5	12.73	4.63	1H-Benz[de]isoquinoline-1,3(2H)-dione
6	12.87	2.41	4-Chloro-6-methoxy-3-quinolinemethanol 9
7	13.01	2.00	1,2-Benzenedicarboxylic acid
8	13.64	79.66	12-Ethylsophoramine
9	14.63	2.86	7,8-Dihydro-14-hydroxymorphinone
10	18.64	1.47	Stigmasterol
11	19.45	0.98	beta.-Sitosterol

Table 5 The EC_{50} value and the R_{max} value obtained for each *Crinum amabile* leaf extract, on phenylephrine pre-contracted endothelium-intact rat aortic rings, using the rat aortic ring assay

Extracts	R_{max} (%)	EC_{50} (mg/mL)
Petroleum extract (PE)	92.65 ± 4.52	0.67
Chloroform extract (CE)	94.72 ± 6.04	0.22
Methanol extract (ME)	66.92 ± 8.16	1.41
Water extract (WE)	65.37 ± 7.71	3.48

All rings were treated with cumulative concentrations ranging from 0.16 to 9.84 mg/mL. All R_{max} values were expressed as mean ± SEM, calculated from eight aortic rings, at the highest cumulative concentration tested

*Treated at cumulative concentration: 4.84 mg/mL

Table 6 The EC_{50} value and the R_{max} value obtained for each fraction of *Crinum amabile* leaf CE, on phenylephrine pre-contracted endothelium-intact rat aortic rings, using the rat aortic ring assay

Fractions	R_{max} (%)	EC_{50} (µg/mL)
F1	96.04 ± 7.05	106.37
F2	87.89 ± 4.75	94.76
F3	82.12 ± 4.27	91.12
F4	85.63 ± 2.83	140.24
F5	100.07 ± 4.28	81.73
F6	45.74 ± 4.06	499.54

All rings were treated with cumulative concentrations ranging from 15.63 to 484.38 µg/mL. All R_{max} values were expressed as mean ± SEM, calculatedfrom eight aortic rings, at the highest cumulative concentration tested. Fraction (F)

*Treated at cumulative concentration: 484.36 µg/mL

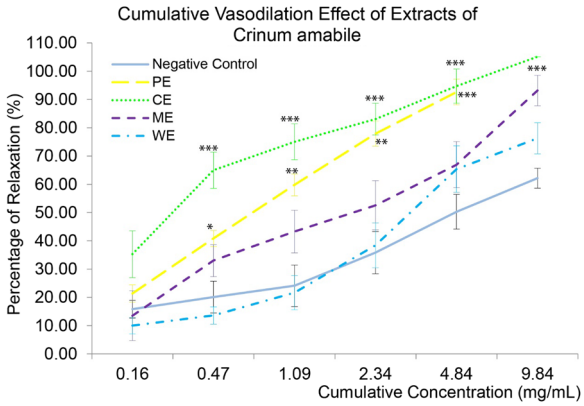

Fig. 4 Vasorelaxation effects of *Crinum amabile* leaf extracts on phenylephrine pre-contracted endothelium-intact rat aortic rings, in the rat aortic ring assay. All aortic rings were treated with cumulative concentrations of extracts ranging from 0.16 to 9.84 mg/mL. Each result represented an average value from eight aortic rings preparations. Values are expressed as mean ± SEM at each cumulative concentration. Significance level was defined as: level 1 $p < 0.05$, level 2 $p < 0.01$ and level 3 $p < 0.001$, as compared to negative control. Petroleum ether extract (PE); chloroform extract (CE); methanol extract (ME) and water extract (WE)

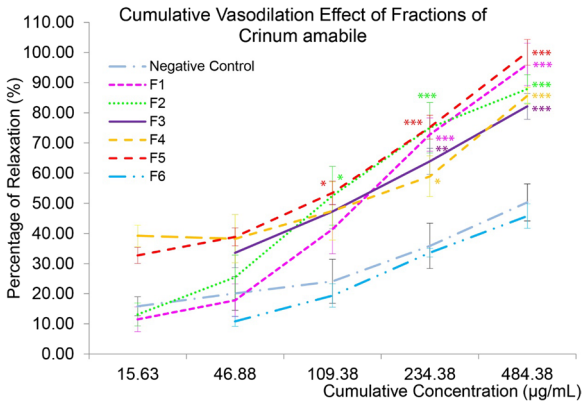

Fig. 5 Vasorelaxation effects of *Crinum amabile* leaf CE fractions on phenylephrine pre-contracted endothelium-intact rat aortic rings, in the rat aortic ring assay. All rings were treated with cumulative concentrations of extracts ranging from 15.63 to 484.38 µg/mL. Each result represented an average value from eight aortic rings preparations and expressed as mean ± SEM at each cumulative concentration. Significance level was defined as: level 1 $p < 0.05$, level 2 $p < 0.01$ and level 3 $p < 0.001$, as compared to negative control. Fraction (F)

4 Discussion

Vasorelaxation can be achieved either by endothelium-dependent mechanisms or through endothelium-independent pathways. While endothelium-dependent vasorelaxation requires an intact layer of the endothelium to secrete endothelium-derived-vasodilators such

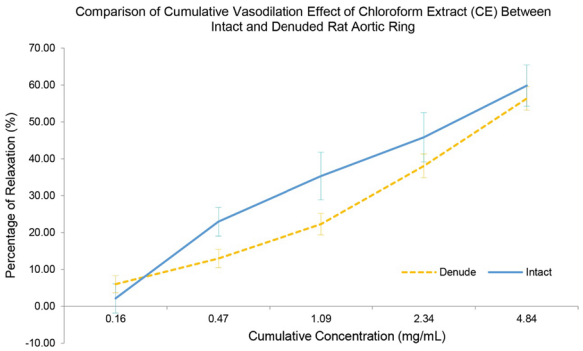

Fig. 6 Vasorelaxation effects of CE on endothelium-intact and endothelium-denuded rat aortic rings. Each result represented an average value from eight aortic rings preparations and expressed as mean ± SEM at each cumulative concentration

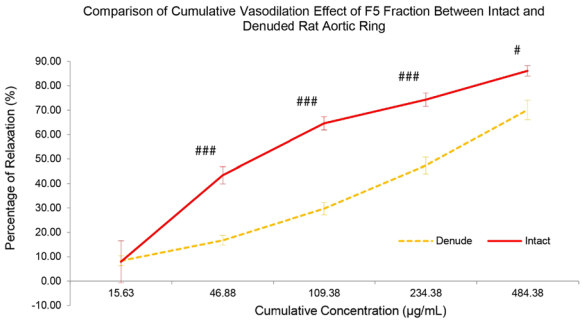

Fig. 7 Vasorelaxation effects of F5 fraction of *Crinum amabile* leaf CE on endothelium-intact and endothelium-denuded rat aortic rings. Each result represented an average value from eight aortic rings preparations and expressed as mean ± SEM at each cumulative concentration. Significance level was defined as: level 1 $p < 0.05$, level 2 $p < 0.01$ and level 3 $p < 0.001$

Table 7 The respective EC_{50} value, R_{max} value and p value obtained for the F5-induced endothelium-dependent vasorelaxation effects in the presence and absence of each respective antagonist and blocker (atropine, L-NAME, methylene blue, propranolol and indomethacin), on phenylephrine pre-contracted endothelium-intact rat aortic rings

MOA	R_{max} (%)	EC_{50} (µg/mL)	*p* value
L-Name	73.47 ± 4.74	231.17	–
Methylene Blue	87.78 ± 4.87	206.76	–
Indomethacin	57.60 ± 3.42	394.32	0.000
Atropine	85.83 ± 4.75	96.72	–
Propanolol	75.18 ± 3.69	83.13	–
Intact	86.11 ± 2.14	77.60	–
Denude	70.11 ± 4.00	300.11	0.036

All rings were treated with cumulative concentrations ranging from 15.63 to 484.38 µg/mL. All R_{max} values were expressed as mean ± SEM, calculated from eight aortic rings, at the highest cumulative concentration tested

*Treated at cumulative concentration: 484.375 µg/mL

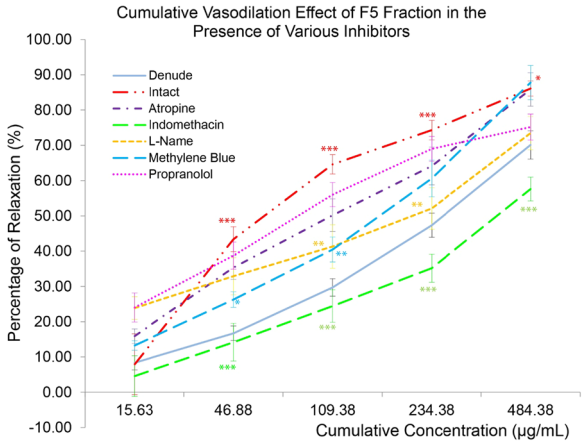

Fig. 8 Endothelium-dependent vasorelaxation effects of F5 fraction of *Crinum amabile* leaf CE on phenylephrine pre-contracted endothelium-intact rat aortic rings in the presence and absence of receptor antagonists and blockers (atropine, L-NAME, methylene blue, propranolol and indomethacin). Each result represented an average value from eight aortic rings preparations and expressed as mean ± SEM at each cumulative concentration. Significance level was defined as: level 1 $p < 0.05$, level 2 $p < 0.01$ and level 3 $p < 0.001$

as NO or PGI_2 [29], endothelium-independent vasorelaxation focuses on the suppression of contractile apparatus (e.g., stimulation of myosin light chain phosphatase (MLCP) activity by a 16-kDa telokin protein, which dephosphorylates the myosin light chain (MLC) and thereby inhibiting muscle contraction while promoting muscle relaxation [30–32] or direct removal of cytosolic calcium ions (Ca^{2+}) [32]. The increase of Ca^{2+} level is usually acquired from two major sources: (1) influx of extracellular Ca^{2+} through transmembrane voltage-operated Ca^{2+} channels (VOCC) or receptor-operated Ca^{2+} channels (ROCC) located in the plasma membrane, and (2) the release of Ca^{2+} from intracellular stores through ryanodine receptor (RyR) or inositol 1,4,5-triphosphate receptor (IP_3R) on sarcoplasmic reticulum (SR) [30–34]. One of the mechanisms involved is through the activation of Ca, Mg-ATPase on the SR [32]. This mechanism is energy-dependent which requires ATP hydrolysis and the binding of magnesium ion (Mg^{2+}) to the catalytic site of the ATPase. Upon activation (phosphorylation), the Ca, Mg-ATPase will bind and translocate two Ca^{2+} into the luminal side of the SR. Besides that, Ca^{2+}-binding proteins such as calsequestrin and calreticulin that are present in SR also help to decrease intracellular Ca^{2+} level. In addition, the plasma membrane of vascular smooth muscle cells (VSMCs) also contains the Ca-Mg-ATPases and the sodium-calcium ($Na^{+}Ca^{2+}$) exchanger, which further facilitate the removal of cytosolic Ca^{2+}. Other mechanisms involved the inhibitions of VOCC or ROCC by channel antagonists such as dihydropyridines, phenyl alkylamines, and benzothiazepines to block the Ca^{2+} entry into the VSMCs [32].

In this study, the vasorelaxation activities and possible underlying mechanism(s) of *Crinum amabile* leaf extracts and fractions were evaluated using the *in-vitro* rat aortic ring assay [19]. The assay was designed based on the contraction-relaxation concept of the blood vessel in an organ-bath system [35]. Since this model integrates the benefits of both *in-vitro* and *in-vivo* models, it offers many advantages in terms of adaptability, reliability, flexibility, simplicity, quickness and cost-effectiveness [19, 27]. It was documented as the most common, yet well-established and excellent model used in vascular function studies [10, 19, 21, 27, 35].

In our study, only those aortic rings with > 50% intact endothelium were [36]. This is crucial since it was highlighted that endothelium is one of the major key elements in vasorelaxation [37]. It is therefore important to preserve the endothelium integrity of the aortic rings as intact as possible. Results from this study showed that CE appeared to produce the highest vasorelaxation activity among all the other *Crinum amabile* leaf extracts. Since vasorelaxation can occur through endothelium-dependent and/or endothelium-independent mechanisms [38, 39], CE was further tested using endothelium-denuded rat aortic rings to investigate whether the vasorelaxation caused by CE was endothelium-dependent or not. Although there was a slight increase in the EC_{50} value (Fig. 3), the removal of endothelium did not significantly abolish the vasorelaxation caused by CE. Nonetheless, it was hypothesized that the amount/ratio of vasoactive constituents present in CE was too low to cause a significant endothelium-dependent vasorelaxation effect. Fractionation of CE was, therefore, necessary to further separate its components. Among the fractions of CE, the F5 fraction exhibited the highest vasorelaxation activity in response to phenylephrine pre-contracted endothelium-intact rat aortic rings, as compared to other fractions. Furthermore, it was demonstrated that the removal of endothelium significantly attenuated the vasorelaxation induced by the F5 fraction suggesting the involvement of endothelium-dependent vasoactivity of the fraction (Fig. 4).

It was worth noting that the total percentage of DMSO accumulated was 0.5%, which was considered high in such an experiment. This concentration of DMSO was able to cause a minor vasorelaxation effect on the rat aortic rings. Therefore, it is always advisable to keep the concentration of DMSO below 0.05% [40, 41] although there was a report on the final cumulative concentration of 1% DMSO used [42]. However, in our experiment, low concentration of extracts, i.e., below 0.16 mg/mL (equivalent

to 0.016% of DMSO) did not cause any significant vasorelaxation effect. Therefore, a high concentration of the extract was tested to determine the vasorelaxation activities of the four extracts. Since our preliminary objective was to compare the vasorelaxation activities among the extracts and fractions, the percentage of DMSO used was considered negligible.

Endothelium-dependent vasodilatation is an important element which forms the basis of vasodilation response [43]. Thus, the possible underlying endothelium-dependent vasorelaxation mechanism(s) of F5 fraction was investigated by pre-incubating the endothelium-intact rat aortic rings with various receptor antagonists or blockers before the administration of F5 fraction. The experiment was designed in such a way as to include most of the important and dominant mechanisms of actions that might involve in the endothelium-dependent vasorelaxation of F5 fraction. These include the NO/sGC/cGMP signalling pathway, the PGI_2 signalling pathway, and the activation of muscarinic and β-adrenergic receptors.

From the results, the application of L-NAME (10 μM) slightly reduced the vasorelaxation caused by F5 fraction with a significant effect occurring at cumulative concentrations of 109.38 μg/mL ($p < 0.01$) and 234.38 μg/mL ($p < 0.01$) (Fig. 5). In addition, the presence of L-NAME also significantly tripled the EC_{50} value compared to normal F5-treated aortic rings (Table 4). This result confirmed the involvement of NO in the F5-induced endothelium-dependent vasorelaxation effect at low concentrations. However, administration of L-NAME did not eliminate the F5-induced vasorelaxation completely, suggesting the involvement of another vasorelaxation mechanism (s). We, therefore further our investigation into the involvement of cGMP in the vasorelaxation mechanism of F5 fraction. Previous studies described both soluble guanylate cyclase (sGC) and cGMP belong to the same NO signalling cascade, in which NO activated sGC while sGC triggered the formation of cGMP from guanosine triphosphate (GTP) [20, 27]. This combined NO/sGC/cGMP mechanism has been regarded as one of the primary and important vasorelaxation pathways involved in vascular smooth muscle [25, 28, 40, 44]. From the results, the endothelium-dependent vasorelaxation effect induced by F5 fraction was found to be fairly affected by incubation with MB (10 μM), with a large increase in EC_{50} value compared to normal F5-treated aortic rings (Table 4). The application of MB only attenuated the F5-induced vasorelaxation from a mild to moderate extent, which was only significant at cumulative concentrations of 46.88 μg/mL ($p < 0.05$) and 109.38 μg/mL ($p < 0.01$) (Fig. 5). The result thus suggested the partial involvement of cGMP in F5-induced endothelium-dependent vasorelaxation at low concentrations. Considering both results, it was proposed that the endothelium-dependent vasorelaxation of F5 fraction was most likely mediated through the NO/cGMP pathway, at least at low concentration. Nevertheless, the inhibitory effects of L-NAME and MB were overcome by the high concentration of F5 fraction suggesting the existence of endothelium-independent vasorelaxation, but this would require further confirmation.

Endothelium-dependent vasorelaxation is closely related to the release of endothelium-derived relaxing factors (EDRFs) such as NO and PGI_2 from the endothelium. Thus, the influence of PGI_2 in the endothelium-dependent vasorelaxation of the F5 fraction was subsequently investigated. We found that the endothelium-dependent vasorelaxation of the F5 fraction was strongly suppressed in the presence of indomethacin (10 μM). The reduction in relaxation in the presence of indomethacin was significant at all cumulative concentrations tested except the lowest concentration (Fig. 5). Although the significance level decreased as the concentration of F5 fraction increased, the above finding implied that the PGI_2 signalling pathway was significantly involved in the F5-induced endothelium-dependent vasorelaxation.

Finally, pre-incubation of the aortic rings with atropine (1 μM) and propranolol (1 μM) failed to cause any significant effect on the F5-induced vasorelaxation in either EC_{50} value, R_{max} value or its concentration–response curve, at all the cumulative concentrations tested as compared to normal F5 treatment (Table 4 and Fig. 5). It is therefore suggested that activation of muscarinic and β2-adrenergic receptors was unlikely to be involved in the endothelium-dependent vasorelaxation activity of F5 fraction.

5 Conclusion

F5 fraction of *Crinum amabile* leaves CE was shown to be the most vasoactive fraction among others, with the highest concentration-dependent vasorelaxation effect on phenylephrine pre-contracted endothelium-intact rat aortic rings. A further assay using phenylephrine pre-contracted endothelium-denuded rat aortic rings concluded that the vasorelaxation induced by F5 fraction was partially endothelium-dependent at low concentrations. In-depth mechanism studies proposed that stimulation of PGI2 production was primarily responsible for the F5-induced endothelium-dependent vasorelaxation, followed by partial association with the NO/cGMP pathway. Other mechanisms such as activation of muscarinic and β-adrenergic receptors played no role in the F5-induced endothelium-dependent vasorelaxation. Nevertheless, further studies are warranted for a complete and detailed understanding of the mechanism(s) of vasorelaxation by

F5 fraction. Based on this study, it can be hypothesized that the F5 fraction might exert a direct vasorelaxation effect on the VSMCs or the aortas, but further investigations are necessary for confirmation. From the current findings, we deduced that the F5 fraction of *Crinum amabile* leaves CE may contain potential vasoactive compounds. *Crinum amabile* can therefore be proposed as a promising candidate for future development of vasodilator drugs which can be used to treat diseases like hypertension, angina, heart disease and so on.

Abbreviations

L-NAME	L-NG nitro arginine methyl ester
MB	Methylene blue
CE	Chloroform extract
PGI_2	Prostaglandin
NO/cGMP	Nitric oxide cyclic guanosine monophosphate
NO	Nitric oxide
EDHF	Endothelium-derived hyperpolarization factor
USM	Universiti Sains Malaysia
UPM	Universiti Putra Malaysia
PE	Petroleum ether extract
ME	Methanol extract
WE	Water extract
DMSO	Dimethyl sulfoxide
TLC	Thin layer chromatography
COX	Cyclooxygenase
SEM	Standard error mean
ANOVA	One-way analysis of variance
MLCP	Myosin light chain phosphatase
MLC	Myosin light chain
VOCC	Voltage-operated Ca^{2+} channels
ROCC	Receptor-operated Ca^{2+} channels
RyR	Ryanodine receptor
IP_3R	Inositol 1,4,5-triphosphate receptor
SR	Sarcoplasmic reticulum
VSMCs	Vascular smooth muscle cells
EDRFs	Endothelium-derived relaxing factors

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Author contributions
C.P.L—Conceptualisation, project administration, methodology; and writing of original draft. R.B.—Conceptualisation, resources; supervision, review and editing of the manuscript. Y.M.F, M.Z.A, V.K.C, N.H.K, Y.K.Y and M.G.L—Data curation, validation, review and editing of the manuscript. All the authors read and approved the final draft of draft of the manuscript.

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Availability of data and materials
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval for the use of animals
All experimental protocols were conducted in accordance with the rules and regulations set by the Institutional Animal Care and Use Committee of UPM under AUP No: UPM/IACUC/AUP-R048/2017.

Consent for publication
Not applicable.

Competing interest
The authors report there are no competing interests to declare.

For studies involving plants
Fresh leaves of *Crinum amabile* were collected from the vicinity of Universiti Sains Malaysia (USM). The plant was authenticated, confirmed and a voucher specimen was deposited at the Biodiversity Unit, Institute of Bioscience, Universiti Putra Malaysia (UPM), under the number SK 3365/18.

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