Hibiscus acid from *Hibiscus sabdariffa* (Malvaceae) has a vasorelaxant effect on the rat aorta

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Abstract

Hibiscus sabdariffa (Malvaceae) is a plant that is widely recognised for its antihypertensive properties; however the constituent(s) responsible for this biological activity are presently unknown. The aim of this study was to identify the potential compounds that are responsible for the vasorelaxant activity of *H. sabdariffa*. Thereafter, the mechanisms involved in producing the vasorelaxation were investigated. The plant was extracted consecutively with hexane, ethyl acetate and methanol. The methanolic extract was subjected to bioassay-guided fractionation in order to isolate pure compounds that possessed vasorelaxant activity. The vascular effects of the pure compounds were studied on the rat aorta *in vitro* using myography techniques. Hibiscus acid produced a concentration-dependent relaxation of the rat aorta pre-contracted with either phenylephrine (3 µM) or KCl (60 mM), irrespective of the presence of the endothelium. When the tissue was pre-contracted with phenylephrine, the concentration required to produce 50% relaxation (IC_{50}), was 0.09 ± 0.01 mg/ml. Hibiscus acid had no effect on the phasic contraction induced by phenylephrine in Ca^{2+}-free physiological solution; but it did affect the component of the contraction that is due to Ca^{2+} influx. In parallel studies, garcinia acid, a diastereoisomer of hibiscus acid, was found to have an almost identical vasorelaxant effect. The vasorelaxant action of both compounds is most likely due to the inhibition of Ca^{2+} influx via voltage-dependent Ca^{2+} channels.

Keywords: *Hibiscus sabdariffa*; Hibiscus acid; Garcinia acid; Vascular smooth muscle; Vasorelaxation; Ca^{2+} channels.

1. Introduction

The red calyces of *Hibiscus sabdariffa (H. sabdariffa)* (from the family Malvaceae), commonly known as Karkade or Red sorrel, have been used in many countries throughout the world for their health benefits, particularly as a treatment for hypertension [1-3]. The antihypertensive activity of *H. sabdariffa* has also been studied clinically on patients with high blood pressure. These studies have shown that regular daily consumption of hibiscus tea caused a clinically significant reduction of approximately 10% in both systolic and diastolic blood pressure [4-9]. The magnitude of the effect of hibiscus tea was similar to that obtained with the angiotensin converting enzyme inhibitors captopril or lisinopril [5, 6].

A number of *in vivo* studies have also shown the blood pressure reducing activity of *H. sabdariffa* calyces in hypertensive animals [10-13]. For example, in salt-induced hypertensive rats, the aqueous extract of the calyxes lowered both systolic and diastolic blood pressure by 6 and 7 mmHg, respectively [13]. There have also been a number of *in vitro* studies examining the vasorelaxant effects of the crude extracts of *H. sabdariffa*. Both aqueous and alcohol extracts of hibiscus calyces produced vasorelaxation of the aorta pre-contracted with either an adrenoceptor agonist or by depolarization [14-18]. The vasorelaxation was proposed to occur through both direct and indirect mechanisms; specifically, by blocking Ca^{2+} entry in smooth muscles cells and by stimulation of endothelial-derived relaxing factor respectively [17, 18]. These crude extracts have also been shown to have inhibitory effects on the contractility of other smooth muscle tissues including the intestine, uterus, and bladder [19, 20].

Phytochemical studies on the calyces have shown that they contain a number of organic acids (hydroxycitric and hibiscus), flavonoids (quercetin, hibiscetin, and gossypetin), and

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anthocyanins (hibiscin) [3]. However, the compounds which are responsible for the antihypertensive activity of *H. sabdariffa* are still unclear. Therefore, the initial aim of this study was to determine which constituent(s) is (are) responsible for this vasorelaxant activity. Thereafter, the activity of the pure compound was compared to the vasorelaxant activity of the crude methanolic extract of this plant. Subsequent studies were carried out to determine the mechanism of action for the vasorelaxant activity of the pure compound on the rat aorta.

2. Materials and Methods

2.1. Plant materials

H. sabdariffa (Malvaceae) was provided by Professor John Igoli. It was collected from Makurdi in Benue State Nigeria and identified at the Herbarium of the National Institute for Pharmaceutical Research and Development (Abuja, Nigeria) by Dr. Jemilat Ibrahim where a voucher specimen number NIPRD/H/6972 was deposited.

2.2. Extraction and purification procedures

We have carried out extraction of the dry calyces in a sequential manner with n-hexane, ethyl acetate and methanol (72 h each) using a Soxhlet apparatus. The extract was then fractionated using chromatographic techniques, and compound identification was carried out using NMR spectroscopy, Mass spectroscopy, and X-ray crystallography [21]. The methanolic crude extract of *H. sabdariffa* calyces was then dissolved in a small volume of methanol and loaded to the top of a Sephadex® LH20 column (GE Healthcare, UK). The column was then eluted with methanol, yielding 5 subfractions (HS-F1 (6%), HS-F2 (25%), HS-F3 (29%), HS-F4 (12%), and HS-F5 (6%)). A preliminary bioactivity-led fractionation process was carried out to examine the vasorelaxant activity of these subfractions using myography techniques (*See section 2.4 below*), where subfractions (HS-F2, HS-F3, HS-F4, and HS-F5) were found to cause vasorelaxation of the aorta. NMR analysis showed that subfractions HS-F2, HS-F3, and

HS-F4 are enriched with hibiscus acid and its derivatives, hibiscus acid methyl ester and hibiscus acid dimethyl ester, while HS-F5 was almost pure hibiscus acid. This result led us to isolate and further purify hibiscus acid, which was prepared in a purity of $\geq 95\%$ (Figure 1A) from the calyces as described previously [21]. Hibiscus acid is not commercially available; however, it is a chiral compound and its diastereomer, garcinia acid (GA) from (*Garcinia cambogia*) is commercially available, (Figure 1B). Therefore, we have carried out parallel studies with garcinia acid, in order to determine if it had a similar vasorelaxant effect to hibiscus acid.
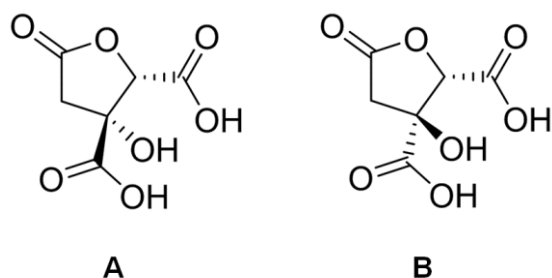
Figure 1: **Structures of hibiscus acid (A) and garcinia acid (B).**

2.3. Drugs, solvents and chemicals

(+)-Garcinia acid, R-(-)-phenylephrine hydrochloride, 1, 4-dihydro-2,6-dimethyl-5-nitro-4-(2-[trifluoromethyl]phenyl)pyridine-3-carboxylic acid methyl ester ((±)-Bay K8644), tetraethylammonium chloride, and carbamylcholine chloride were all purchased from Sigma-Aldrich (Gillingham, UK). 2,5-dimethyl-4-[2-(phenyl methyl) benzoyl]-1H-pyrrole-3-carboxylic acid methyl ester (FPL 64176) was obtained from Tocris (Abingdon, UK). All other reagents and salts used were from either VWR chemicals (Lutterworth, UK), or Sigma-Aldrich. The crude extract, hibiscus acid, and garcinia acid were prepared as 20 mg/ml stock solutions, dissolved in physiological salt solution (PSS), of the following composition (in

mM): NaCl 150, KCl 5.4, glucose 10, HEPES 10, $MgCl_2$ 1.2, and $CaCl_2$ 1.8, pH adjusted to 7.4 with NaOH. Solutions of carbamylcholine chloride, tetraethylammonium chloride, and phenylephrine (all 100 mM) were prepared using PSS. Bay K8644 and FPL 64176 were dissolved in dimethyl sulfoxide (DMSO) as 10 mM stock solutions, and subsequent dilutions were made using PSS. In the case of Bay K8644 and FPL 64176, the same dilutions of DMSO were used in the control experiments.

2.4. Aorta tissue preparation and wire myography

In-house bred male and female Sprague-Dawley rats (200-250 g) were sacrificed by cervical dislocation, and all studies were carried out in accordance with the guidelines and principles for the care and use of laboratory animals at the University of Strathclyde according to Schedule 1 of the Animals (Scientific Procedures) Act 1986. The thoracic and abdominal aorta was subsequently removed, and placed in cold PSS. Under a dissecting microscope (Nikon SMZ645), the aorta was cleaned of any adhering fat and loose connective tissue. The aorta was then cut into 4-5 mm long rings and mounted on intraluminal parallel wires; one of which was fixed and the other attached to a Grass FT03C force displacement transducer. The organ bath (1 ml in volume) was filled with PSS and maintained at 37^oC whilst being aerated with air. A resting tension of 1 g was applied to the tissue and a 1 h equilibration period was allowed, with regular reintroduction of fresh PSS and re-adjustment of the 1 g resting tension. The contractile response was acquired through a PowerLab 4/30 data acquisition system and recorded by Chart (v5.2) software (ADInstruments, Ltd., Oxfordshire, UK). Before commencing any experimental protocol the tissue was challenged repeatedly with 40 mM KCl until the contractile responses were reproducible.

2.5. Effects of the crude methanolic extract of *H. sabdariffa* on rat aorta

In order to investigate the vasorelaxant activity, endothelium intact aorta sections were pre-contracted with PE (3 μM) or KCl (60 mM). Once the contractions were stable, 0.001-1 mg/ml crude extract of *H. sabdariffa* was added in a cumulative manner to the tissues pre-contracted with PE, and 0.01-2 mg/ml crude extract was used in the tissues pre-contracted with KCl.

2.6. Effects of hibiscus and garcinia acid on rat aorta

As per the protocol described above for the crude extract, 0.001-1 mg/ml hibiscus or garcinia acid was added in a cumulative manner to the tissues pre-contracted with PE, and 0.01-2 mg/ml hibiscus or garcinia acid was used in the tissues pre-contracted with KCl.

The majority of the experiments were carried out on endothelium-intact aortas, unless otherwise indicated. Confirmation that the endothelium was intact was demonstrated by a greater than 70% relaxation in response to carbachol (10 μM), when the aorta was pre-contracted with PE (3 μM). The influence of the endothelium on the relaxant response of either hibiscus or garcinia acid was examined, and this was established by examining their effect in denuded preparations. The endothelium was denuded by gently rubbing the luminal surface of the aortic ring with a sanded down wooden cocktail stick. The absence of the endothelium was confirmed by less than 10% relaxation in response to carbachol (10 μM), in aorta pre-contracted with PE (3 μM).

2.7. Role of L-type voltage dependent calcium channels (VDCCs) on hibiscus and garcinia acid induced relaxation

To establish whether hibiscus and garcinia acid produce their relaxation by inhibiting L-type voltage dependent calcium channels (VDCCs), their effect on the contractions produced by FPL 64176, a benzopyrrole-type agonist of L-type Ca^{2+} channels [22] or Bay K 8644 [23]

was examined. The tissue was initially depolarised with 20 mM KCl for 5 min, to activate VDCCs and produce a minimal contractile response. Thereafter, FPL 64176 (30 µM) or Bay K 8644 (0.1 µM) was added in order to produce further activation of VDCCs [22, 24]. Once the contractile response had stabilised, either hibiscus acid or garcinia acid, was applied in concentrations 0.5-1 mg/ml.

2.8. Effect of hibiscus and garcinia acid on the release of intracellular Ca^{2+}

A component of the contractile response to PE involves Ca^{2+} release from the sarcoplasmic reticulum (SR), which produces a phasic contractile response [25]. To examine the effect of hibiscus and garcinia acid on SR Ca^{2+} release a control contractile response to PE (3 µM) in regular PSS was initially obtained. After a 1 h recovery period, the tissue was exposed to a Ca^{2+} free physiological solution containing 1 mM ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) for 15 min. During this period, either vehicle, hibiscus or garcinia acid was applied and the tissue was subsequently re-challenged with PE (3 µM).

2.9. Effect of hibiscus and garcinia acid on the influx of extracellular Ca^{2+}

Another component of the contractile response to PE involves Ca^{2+} influx from the extracellular space, which produces the sustained (tonic) phase of the contractile response [25]. To determine the effect of hibiscus and garcinia acid on the tonic phase of contraction, the tissue was exposed to a Ca^{2+} free physiological solution containing 1 mM EGTA for 15 min, the tissue was then challenged with PE (3 µM) for 5 min to produce the phasic contractile response. Thereafter, 1.8 mM Ca^{2+} (producing a free calcium concentration 0.8 mM as calculated using the MAXC computer program), was reintroduced to the bath, resulting in a tonic contraction Hibiscus or garcinia acid (0.5 mg/ml) was applied during the tonic phase of the contraction.

2.10. Effect of blocking potassium channels on the relaxant activity of hibiscus and garcinia acid

To establish whether the relaxation to hibiscus or garcinia acid involves the activation of K^+ channels, their effect was examined in the presence of the non-selective K^+ channel blocker tetraethyl ammonium chloride (TEA) [26]. The aorta was challenged with PE (3 μM) alone, and 0.001 -1 mg/ml hibiscus or garcinia acid was added as described above. Then, and after 1 h recovery, the aorta was preincubated with 6 mM TEA for 20 min prior to pre-contraction with PE (3 μM), and once the contractile response had stabilised, hibiscus or garcinia acid was applied to the tissue in a cumulative manner.

3. Statistical analysis

For the contractile responses, the data were expressed as percentage of maximum contraction to either agonists or KCl. Relaxation of the tissues is expressed as the measured tension following the addition of hibiscus acid or garcinia acid divided by the steady state tension produced by the contractile stimulus being used, x100. Nonlinear regression analysis (GraphPad Prism version 7, San Diego, CA, USA) was used for fitting the log concentration-response curve and determination of the IC_{50} (concentration required to produce 50% of the maximum inhibitory effect) and I_{max} (the maximum inhibitory effect). One tissue that was pre-contracted always served as a control. All data are presented as mean ± standard error of the mean (s.e.m.). The number of observations are expressed in the format of n = *N/n* where *N* is the number of tissues and *n* is the number of animals. One-way ANOVA with Tukey's multiple comparisons test was used to compare the treatments with control groups, and Student's two-tailed unpaired *t*-test was applied for statistical comparison of IC_{50} of different groups. $P<0.05$ was considered to be statistically significant.

4. Results

4.1. Effect of the crude extract of *H. sabdariffa* on the rat aorta

The crude methanolic extract of *H. sabdariffa* produced a concentration-dependent relaxation of endothelium-intact rat aorta pre-contracted with PE (3 μM). The IC_{50} for the crude extract was 0.33 ± 0.04 mg/ml (Figure 2A) and the highest concentration tested (1 mg/ml) relaxed the tissue by $76 \pm 4\%$ (n=12/4). The crude extract also produced a concentration-dependent relaxation of the aorta when it was pre-contracted with KCl (60 mM) with an IC_{50} of 1.5 ± 0.5 mg/ml; n=16/4. At 1 mg/ml the crude extract produced relaxation of $33 \pm 2\%$ when it was applied to the aorta pre-contracted with KCl (60 mM), and this was increased to $68 \pm 2\%$ at the highest concentration examined (2 mg/ml) (Figure 2B).

4.2. Effect of hibiscus and garcinia acid on the rat aorta

Hibiscus acid caused a concentration-dependent relaxation of the aorta pre-contracted with PE (3 μM). The IC_{50} for hibiscus acid was 0.09 ± 0.01 mg/ml (Figure 3A) in the endothelium-intact aorta and the highest concentration tested (1 mg/ml) almost completely relaxed the tissue ($96 \pm 2\%$ relaxation) (n=18/6). The relaxation was maintained for as long as hibiscus acid was present, and the tissue showed complete recovery in response to PE after washing it out and allowing approximately 60 min for recovery. The time control for these experiments showed a slight relaxation ($15 \pm 4\%$) in the PE-induced contraction, over the time course of the experiment.

When the endothelium was removed, the magnitude of contraction produced by PE increased by 27% (n=32/8) compared to that induced in intact aorta. The relaxant effect of hibiscus acid was also examined on endothelium-denuded aorta, where the magnitude of the relaxation was similar ($89 \pm 3\%$) (n=9/6); but there was a 2-fold shift to the right with regard

to the sensitivity to hibiscus acid, yielding an IC_{50} 0.19 ± 0.02 mg/ml (P<0.001 compared to endothelium intact) (Figure 3Aii). It was also evident that the lowest concentration of hibiscus acid tested (1 mg/ml) had no relaxant effect in the denuded aorta, whereas it caused $10 \pm 2\%$ relaxation in the endothelium intact aorta. In tissues that had been denuded of endothelium, which served as a time control, there was a slight increase in the PE-induced contraction of $5 \pm 9\%$ over the course of the experiment.

Garcinia acid was found to have a similar effect to that of hibiscus acid, showing almost complete relaxation in both endothelium intact and denuded aorta. The maximum relaxation to garcinia acid was similar irrespective of whether the endothelium was intact ($94 \pm 2\%$) or denuded ($92 \pm 2\%$). However, the IC_{50} value for garcinia acid was significantly higher in the endothelium denuded aorta (0.2 ± 0.02 mg/ml; n=9/6) compared to the endothelium intact preparation (0.12 ± 0.01 mg/ml; $P < 0.001$; n=18/6) (Figure 3B). In comparing the relaxant activities of both hibiscus and garcinia acids, there was no significant difference between the diastereomers to antagonise the PE-induced contraction.
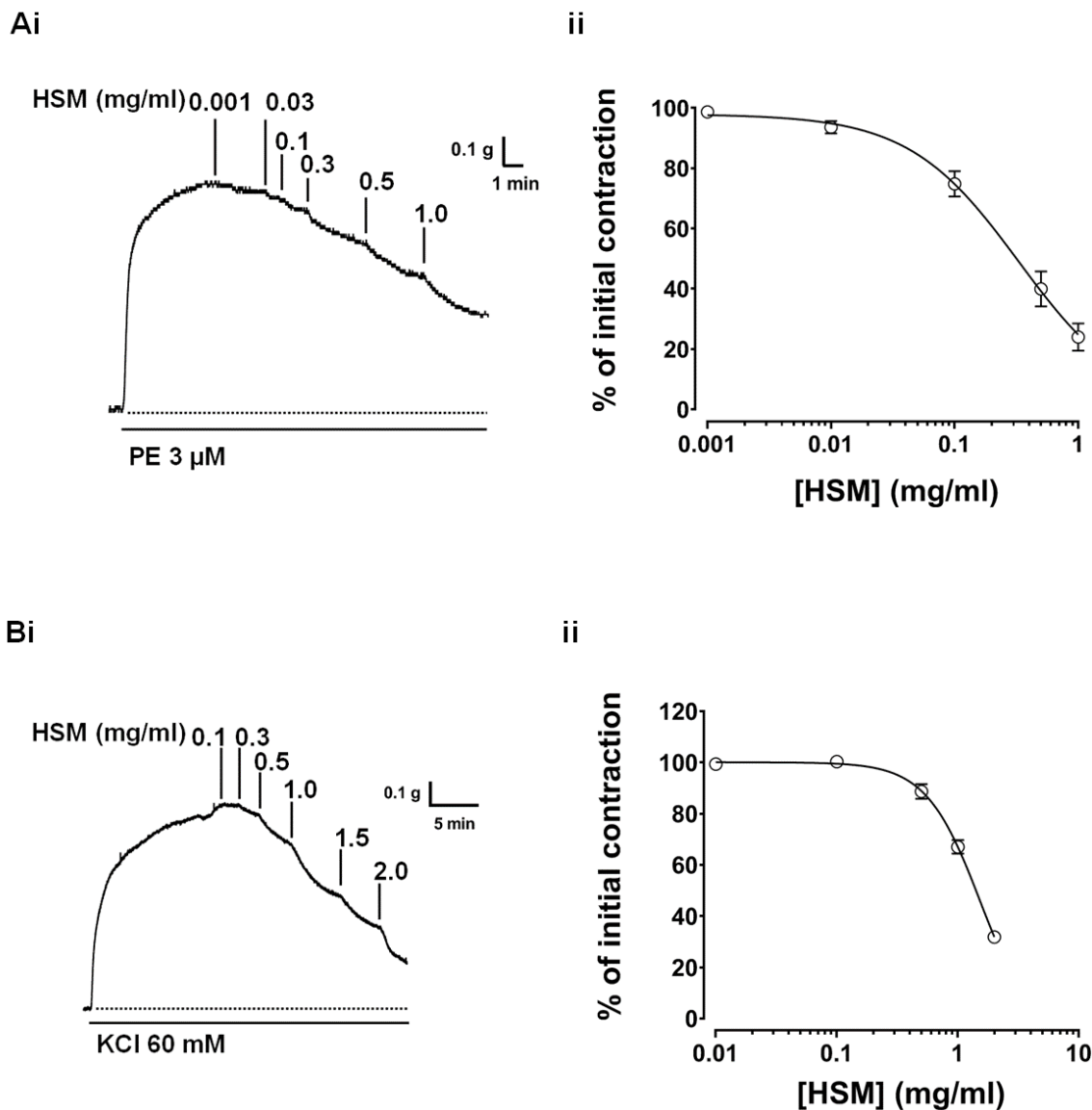
Figure 2: Relaxant effect of the crude methanolic extract of *H. sabdariffa* (HSM) on the rat aorta. Representative recordings of the effect of HSM on endothelium-intact rat aorta pre-contracted with PE (3 μM) (**Ai**), and KCl (60 mM) (**Bi**). Summary figures of the relaxation due to HSM on the aorta pre-contracted with PE (**Aii**), and KCl (**Bii**). The relaxation is expressed as a percentage of the initial contraction. Data represent mean ± s.e.m. (n=12/4 for PE pre-contraction and n=16/4 for KCl pre-contraction).

When the aorta was pre-contacted with KCl (60 mM), hibiscus acid also produced a concentration-dependent relaxation. The IC_{50} was 0.57 ± 0.06 mg/ml; n=12/6 and the highest concentration of hibiscus acid examined (2 mg/ml) produced a relaxation of $77 \pm 5\%$ (Figure 4A). When the endothelium was removed, there was no change in the sensitivity of the aorta to hibiscus acid (IC_{50} was 0.66 ± 0.1 mg/ml; n=6/4) or the magnitude of the relaxation obtained at the highest concentration examined ($72 \pm 6\%$) (Figure 4A).

Similarly, garcinia acid also relaxed the aorta pre-contracted with KCl (Figure 4B). As with hibiscus acid, there was no significant effect of removing the endothelium on the IC_{50} (0.38 ± 0.06 mg/ml; n=13/6 for intact and 0.45 ± 0.08 mg/ml; n=10/4 for denuded), or the magnitude of the relaxation produced by garcinia acid at the highest concentration tested ($77 \pm 5\%$ and $72 \pm 5\%$, respectively). Over the course of these experiments the time control showed a $20 \pm 4\%$ increase in the contractile response to KCl in the endothelium intact preparation and a $22 \pm 3\%$ increase in the endothelium-denuded preparation. There was no significant difference between hibiscus acid and garcinia acid in their ability to relax the KCl contraction. However, the concentration of either hibiscus or garcinia acid that was needed to relax the KCl-induced contractions was significantly higher ($P < 0.01$) than that required to relax the PE-induced contractions.
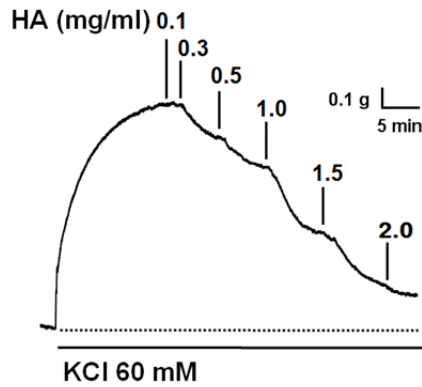
4.3. Relaxant effect of hibiscus or garcinia acid on contraction induced by L- type calcium channel activators

Since hibiscus and garcinia acid relaxed the aorta pre-contracted with KCl, and the contraction induced by KCl is primarily dependent on the activation of voltage-dependent Ca^{2+} channels (VDCCs), one possible mechanism to explain their vasorelaxant activity is through inhibition of VDCCs. To examine this further, the effect of these substances on the contractions induced by Ca^{2+} channel activators was examined.
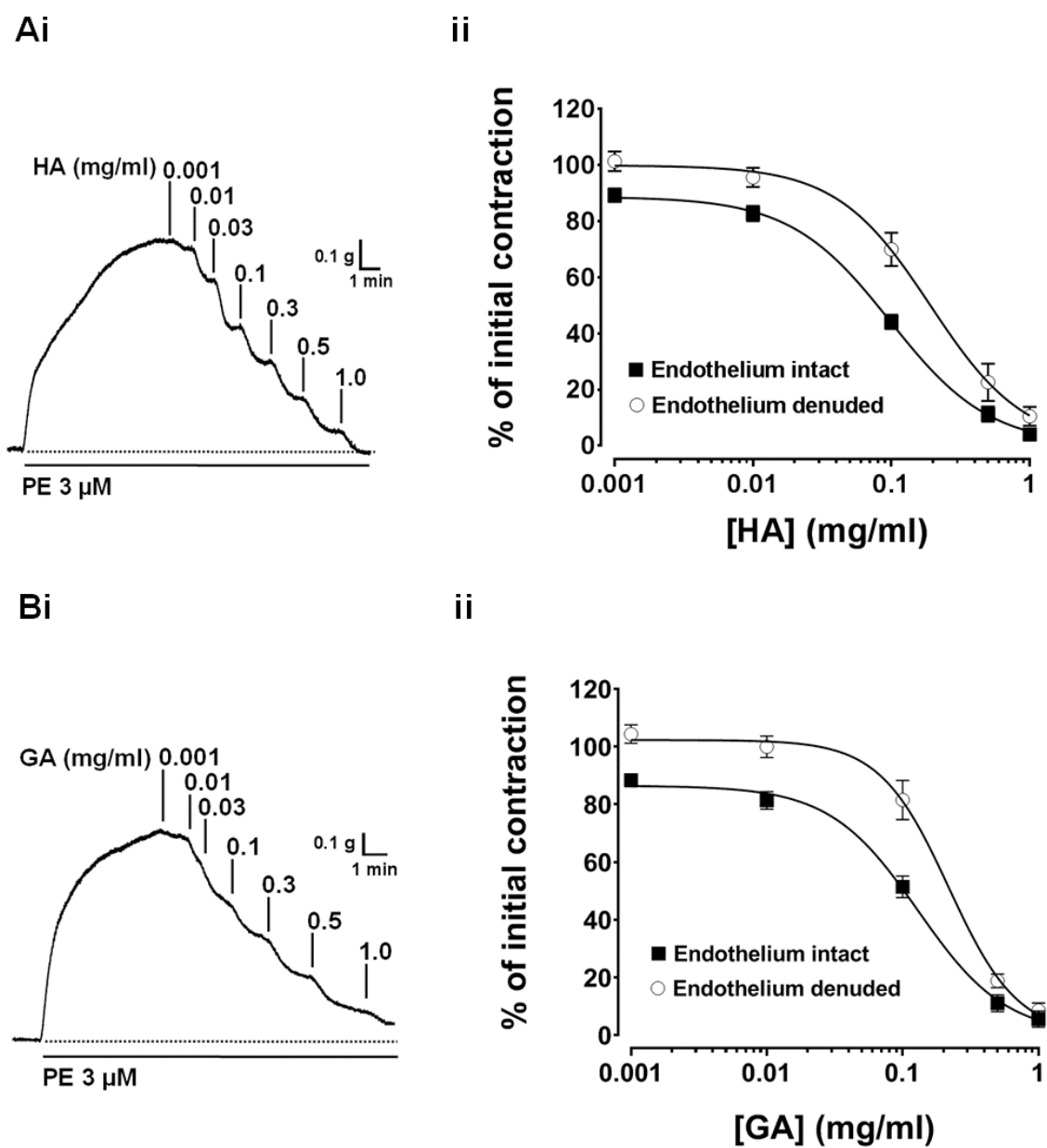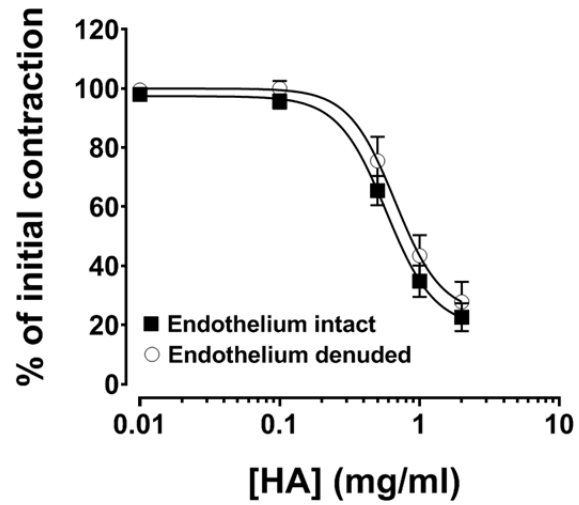
Figure 3: Relaxant effect of hibiscus acid and garcinia acid on the rat aorta pre-contracted with PE. Representative recordings of the effect of HA (Ai) and GA (Bi) on the endothelium-intact aorta pre-contracted with PE (3 μM). Relaxant effect of HA (Aii) and GA (Bii) on PE-induced contraction of endothelium-intact and endothelium-denuded aortic rings. The relaxation is expressed as a percentage of the initial contraction. Data represent mean ± s.e.m. (n=18/6 for the intact aortic rings and n=9/6 for the denuded rings).
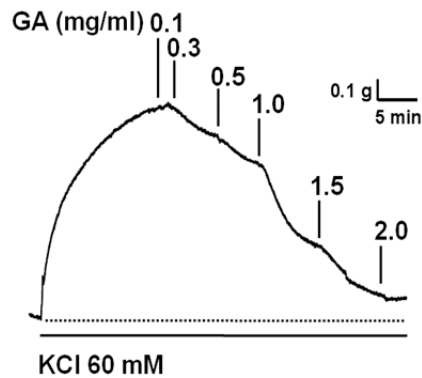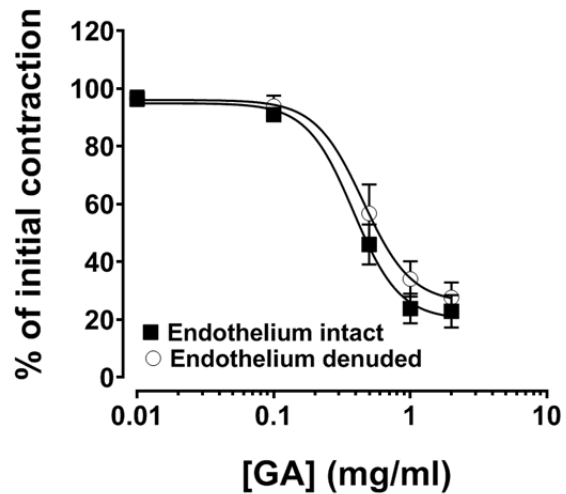
Figure 4: Relaxant effect of hibiscus acid and garcinia acid on the rat aorta pre-contracted with KCl. Representative recording of the effect of HA (Ai) and GA (Bi) on the endothelium-intact aorta pre-contracted with KCl (60 mM). Relaxant effect of HA (Aii) and GA (Bii) on KCl-induced contraction of endothelium-intact and endothelium-denuded aortic rings. The relaxation is expressed as a percentage of the initial contraction. Values represent mean ± s.e.m. (n=12-13/6 for the intact aortic rings and n=6-10/4 for the denuded rings).

All the aortas pre-contracted with a low concentration of KCl followed the addition of either FPL 64176 or Bay K 8644, showed an oscillatory type of contractile response.

When the aorta was pre-contracted with FPL 64176 (30 µM), hibiscus acid (0.5 mg/ml) almost completely relaxed the tissue (95 ± 2% relaxation; n=4/4). A similar effect was observed when Bay K 8644 (0.1 µM) was used to contract the aorta, although in this case a higher concentration of hibiscus acid (1 mg/ml) was required to produce a similar degree of relaxation (94 ± 4%; n=4/4) (Figure 5). The effect of garcinia acid (0.5 mg/ml) was very similar to that of hibiscus acid; producing 94 ± 4% (n=4/4) relaxation of the aorta pre-contracted with FPL 64176 and requiring a higher concentration (1 mg/ml) to almost completely relax the aorta (93 ± 2%; n=4/4) when pre-contracted with Bay K 8644 (Figure 5).
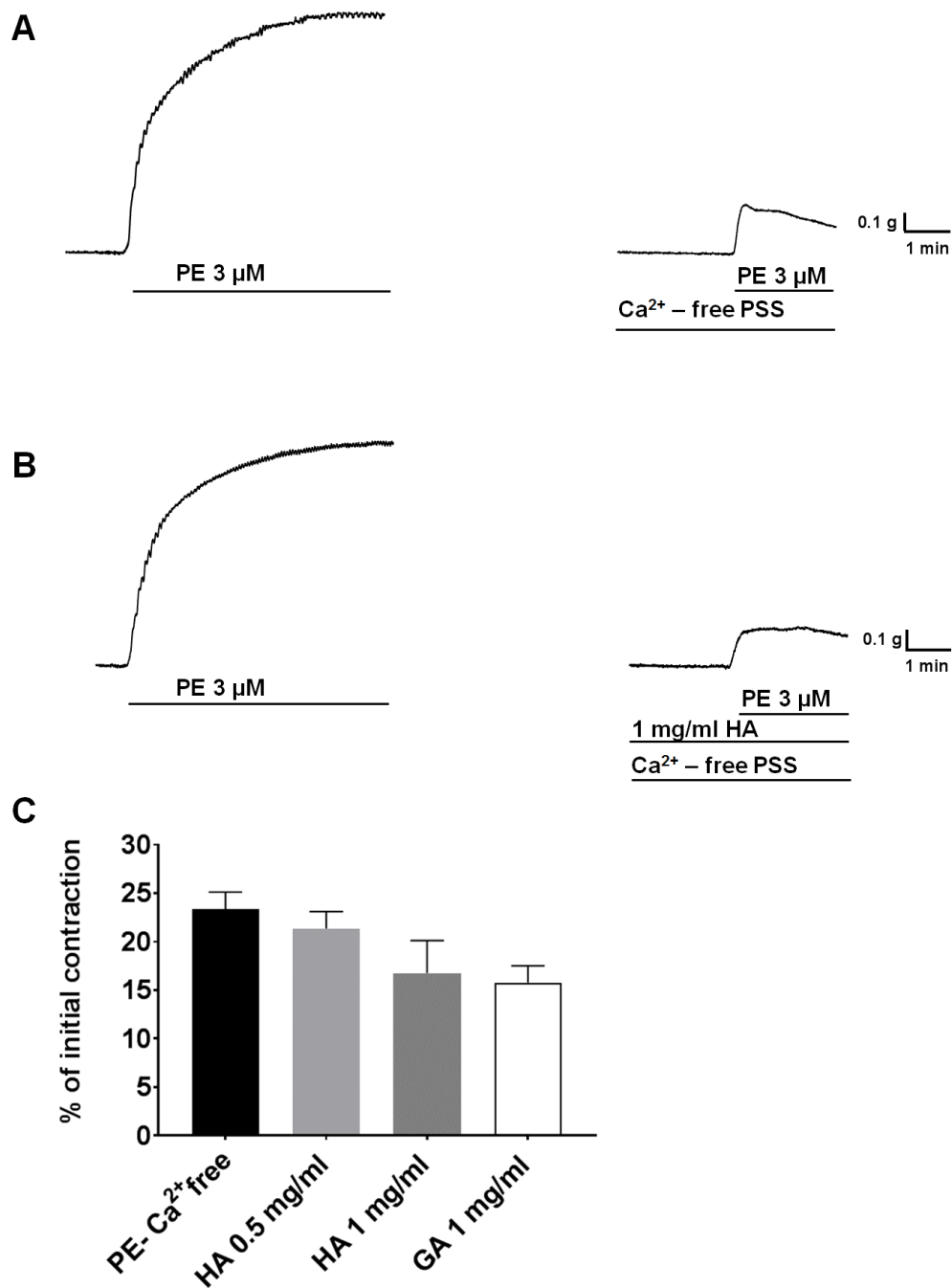
4.4. Effect of hibiscus and garcinia acid on the phasic and tonic contractions induced by PE in the rat aorta

In the absence of extracellular calcium, the phasic contraction of the aorta induced by PE is due to the release of intracellular calcium from intracellular stores [25]. Under Ca^{2+}-free conditions, PE (3 µM) produced a transient contraction, which was 23 ± 1% of the contraction produced by PE in Ca^{2+} containing PSS (Figure 6). Neither hibiscus nor garcinia acid (1mg/ml) had any significant effect on the transient contraction to PE in Ca^{2+}-free PSS, being 16 ± 3% and 15 ± 1% in the presence of the respective acids (n=4/4) (Figure 6). After an initial phasic contraction to PE (3 µM) was obtained in Ca^{2+}-free PSS, this developed into a sustained contraction following the subsequent re-addition of Ca^{2+} to the PSS. When hibiscus acid (Figure 7A) or garcinia acid (Figure 7B) was added during the sustained phase of the contraction, it caused a relaxation of 71 ± 3% and 76 ± 5%, respectively (n=4/4) (Figure 7C).
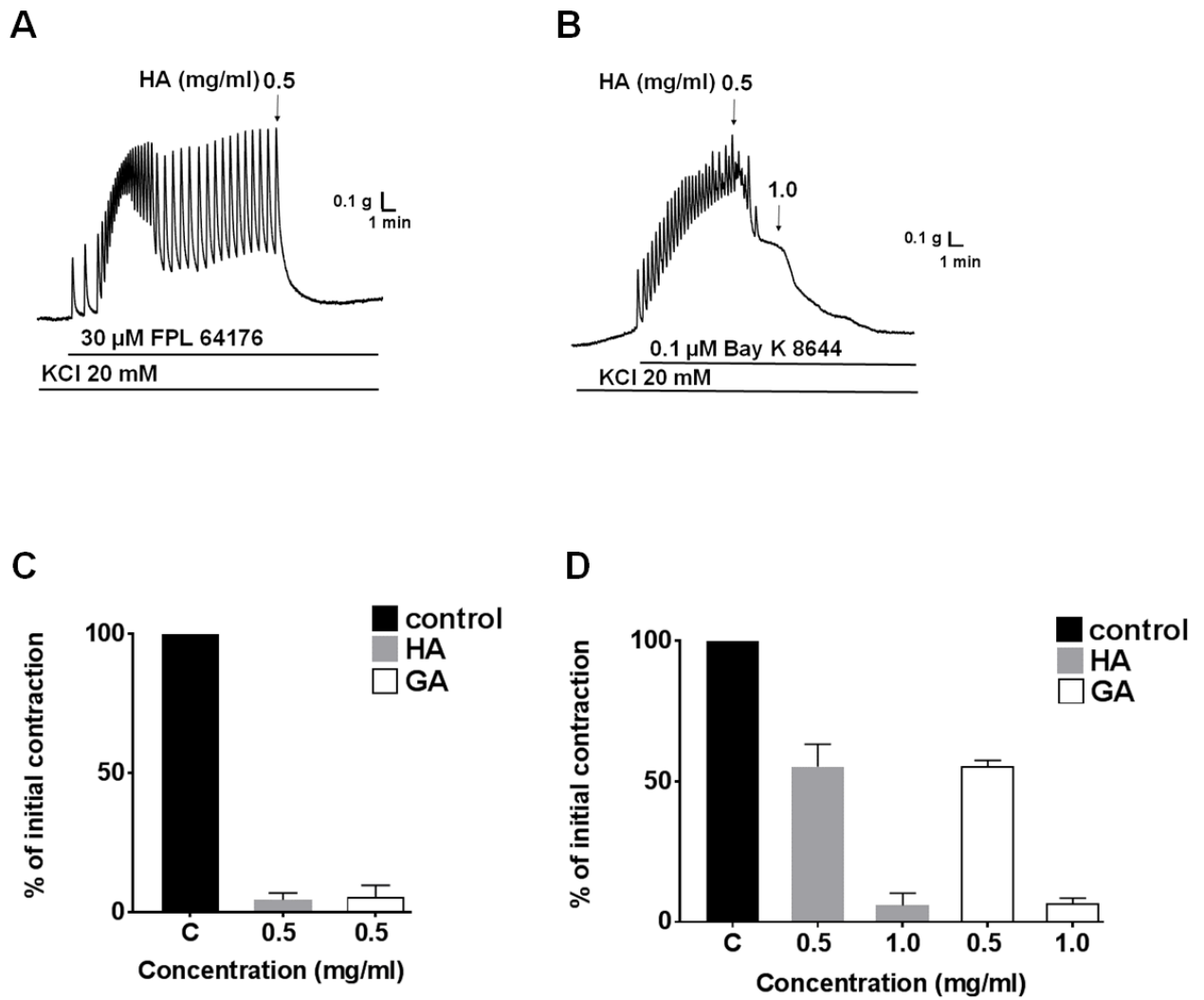
Figure 5: **Relaxant effect of hibiscus acid and garcinia acid, on the rat aorta pre-contracted with L-type calcium channel activators.** Representative recording of the effect of HA (0.5-1 mg/ml) on the contraction induced by 30 µM FPL 64176 (A) or 0.1 µM Bay K 8644 (B). Summary figures of the relaxation due to HA and GA when the aorta was pre-contracted with either FPL 64176 (C) or Bay K 8644 (D). Data represent mean ± s.e.m. (n=4/4).

Figure 6: **Effect of hibiscus acid and garcinia acid on the phasic component of contraction induced by PE (3μM) in the rat aorta.** Representative control recording of the PE-induced contraction in the presence and absence of extracellular Ca^{2+} (A). The effect of HA (1 mg/ml) on the PE-induced contraction in the absence of extracellular Ca^{2+} (B). (C) Summary figure showing the effects of HA (0.5 and 1 mg/ml) and GA (1 mg/ml) on the PE-induced transient contraction of the aorta. Data represent mean ± s.e.m. (n=4/4).
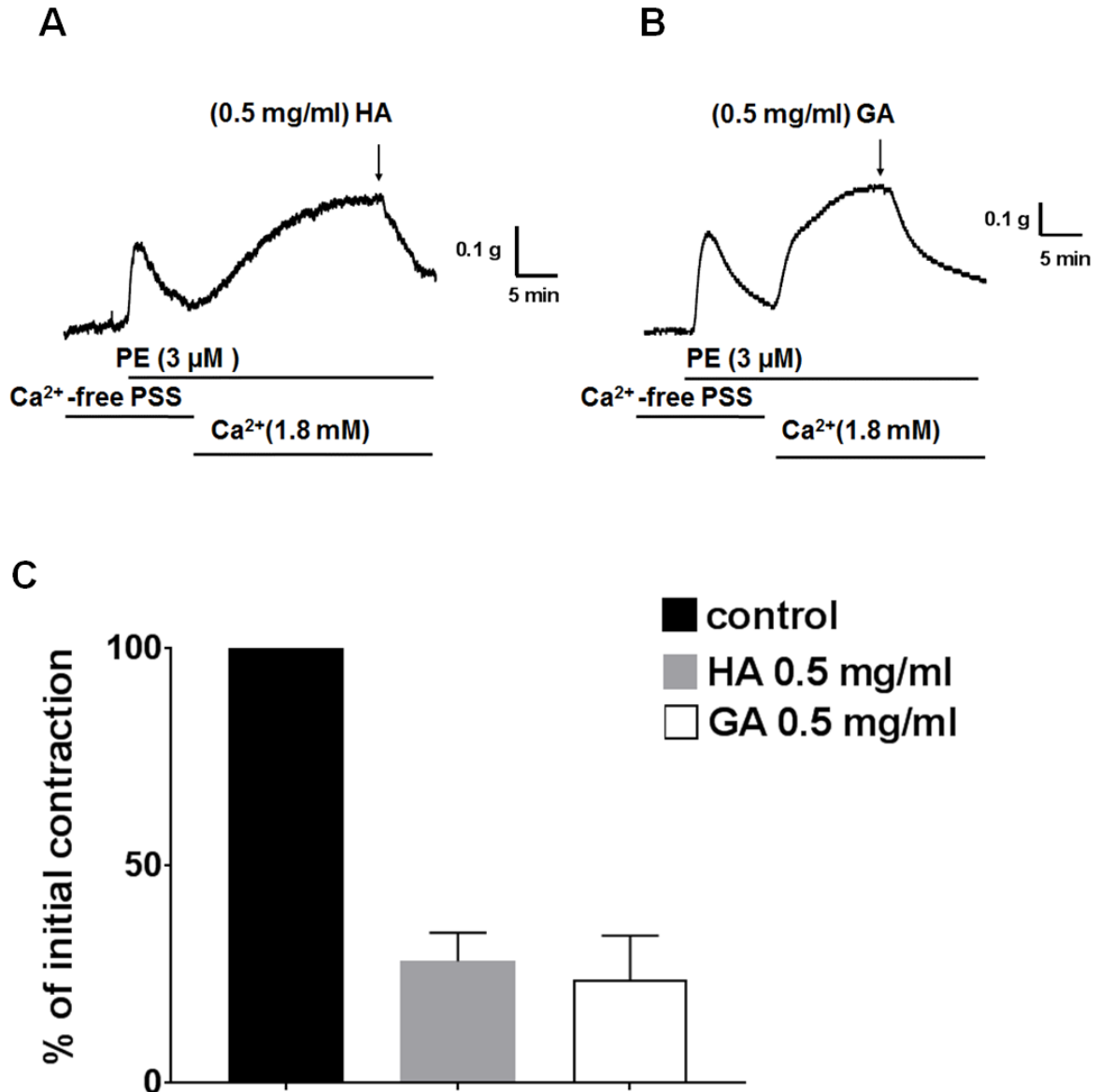
Figure 7: Relaxant effect of hibiscus and garcinia acid on the sustained component of the PE-induced contraction. Representative recordings showing the effect of hibiscus acid (A) and garcinia acid (B) on the tonic component of the PE-induced contraction, following the addition of 1.8 mM Ca^{2+}. The intracellular store was depleted by the application of PE in a Ca^{2+} free medium and the re-addition of Ca^{2+} produced the tonic phase of contraction. (C) Summary figure showing the effects of HA and GA (0.5 mg/ml) on the tonic component of the PE-induced contraction. Values represent mean ± s.e.m. (n=4/4).

4.5. Effect of a potassium channel blocker on the relaxant effect of hibiscus and garcinia acid

Participation of K^+ channels in the relaxant effect of hibiscus or garcinia acid was also examined, using the non-selective potassium channel blocker (TEA). Pre-incubation of the aorta with TEA (6 mM) increased the magnitude of PE-induced contraction by 34% (n=13/7) when compared to that produced in the absence of TEA. The IC_{50} for hibiscus acid induced relaxation of the PE pre-contracted aorta was significantly (P<0.05) increased from 0.09 ± 0.01 mg/ml under control conditions to 0.3 ± 0.04 mg/ml in the presence of TEA, and the maximum relaxation was slightly reduced from 95 ± 4% to 82 ± 5%; n=6/6) (Figure 8A). A similar effect of TEA was observed when garcinia acid was used as the vasorelaxant. Specifically, TEA significantly (P<0.05) increased the IC_{50} for garcinia acid from 0.13 ± 0.02 mg/ml to 0.25± 0.03 mg/ml and reduced the maximum relaxation from 93 ± 4 % to 86 ± 2%; n=7/6) (Figure 8B).
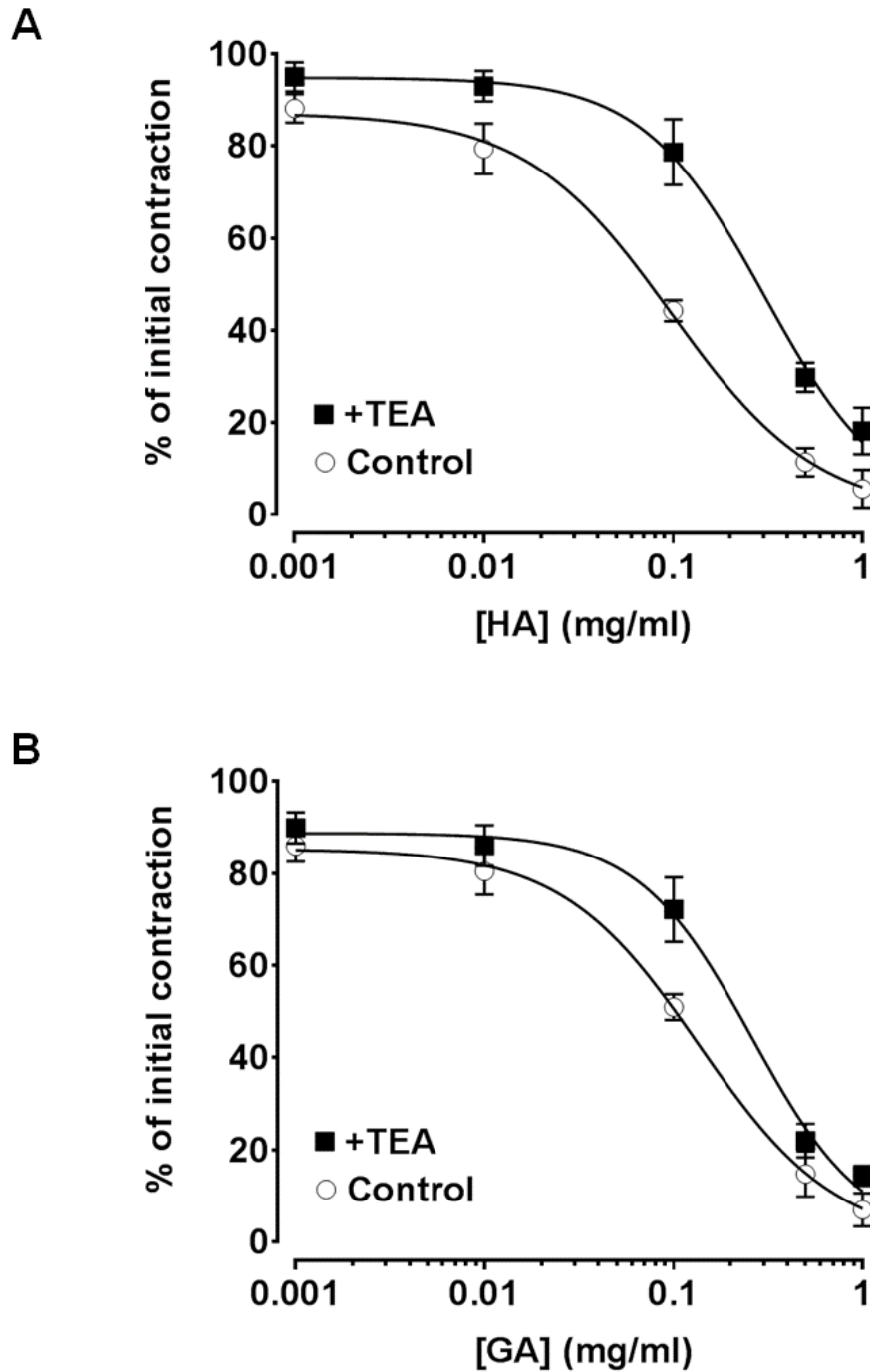
Figure 8: **The effect of potassium channel blockade on the relaxation induced by hibiscus or garcinia acid on the PE pre-contracted rat aorta.** Relaxant effect of HA (A) and GA (B) on the PE pre-contracted endothelium-intact aorta in presence and absence of TEA (6 mM). The relaxation is expressed as a percentage of the initial contraction. Data represent mean ± s.e.m. (n=6/6 for HA and 7/6 for GA).

5. Discussion

There is considerable evidence in the literature that *H. sabdariffa* has an antihypertensive effect and this is further supported by a number of *in vitro* studies demonstrating a vasorelaxant effect of the crude extract of this plant [14-18, 27, 28]. This study demonstrated the vasorelaxant activity of the crude methanolic extract of *H.sabdariffa* calyces on the rat aorta, and it was found to be significantly more potent in its relaxant effect when the tissue was pre-contracted with PE compared to KCl. These results are in agreement with what has been reported previously [17], where the crude methanolic extract at a concentration of 1 mg/ml produced ~30% relaxation when the aorta was pre-contracted with KCl, and ~80% relaxation when it was pre-contracted with PE.

Using a bio-assay guided fractionation process, this study has now shown that hibiscus acid, one of the constituents isolated and identified from the calyces of *H. sabdariffa* has a direct vasorelaxant effect on the rat aorta, which may, at least in part, be the constituent responsible for the vascular activity of this plant. With regard to the vasorelaxant effect of hibiscus acid, it was found to be more potent and efficacious when compared to the activity of the crude extract. The complementary finding that commercially available garcinia acid, which is the main organic acid found in *Garcinia cambogia* (*G. cambogia*) (Clusiaceae), had a very similar effect, provides further support for the notion that hibiscus acid has vasorelaxant activity. Both hibiscus and garcinia acid produced a concentration-dependent relaxation of the aorta when it was pre-contracted with either PE or KCl. Their effects were fully reversed upon their washout indicating that, over the concentration range used, they had no deleterious effect on the tissue. Both hibiscus and garcinia acid were found to be significantly more potent in their relaxant effect when the tissue was pre-contracted with PE, when compared to

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pre-contraction with KCl. A similar finding was observed with the crude methanolic extract of *H. sabdariffa* [17].

Removal of the endothelium did not prevent the relaxation of the aorta to either hibiscus or garcinia acid in the PE pre-contracted aorta, with a similar degree of relaxation being achieved in endothelium intact and endothelium denuded tissues. Whilst this indicates that the relaxation to both these substances is endothelium independent there was nevertheless a slight rightward shift in the concentration-response curve to both hibiscus and garcinia acid after removal of the endothelium. This may indicate a modulatory role of the endothelium in the response to either hibiscus or garcinia acid. However, it may also have been a consequence of the increased contraction produced by PE when the endothelium was removed, a finding that has previously been reported by others [29]. When the aorta was pre-contracted with KCl, there was no effect of removing the endothelium on the relaxation produced by either hibiscus or garcinia acid, and in this case the contractile response to KCl was not significantly affected by removal of the endothelium either. It should be noted that previous studies with the crude extracts of *H. sabdariffa* have found that the relaxation is significantly affected by removal of the endothelium [15, 17, 18], thus, it is clear that there could be additional constituents in the crude extract that are affecting vascular activity.

As both hibiscus and garcinia acid produced relaxation of the rat aorta pre-contracted with KCl, one possible explanation for their observed effect is that they are blocking Ca^{2+} channels. This is because the contraction induced by KCl predominantly involves depolarisation of the smooth muscle cell membrane potential and the resultant activation of VDCCs. Indeed, the relaxation of this type of contraction is frequently utilised when studying the effect of VDCC blockers [24, 30-35]. Further support for this mechanism of action is

provided by the studies showing that hibiscus and garcinia relaxed the aorta when it was pre-contracted with the selective L-type Ca^{2+} channel activator FPL 64176 or Bay K8644 [36, 37]. The contraction induced by FPL 64176 was completely relaxed by lower concentrations of hibiscus or garcinia acid than what was required when Bay K8644 was used to pre-contract the tissue. A possible explanation for the difference in sensitivity may be related to the agonistic properties of FPL 64176 and Bay K8644, as both L-type Ca^{2+} channel activators act via a mechanism and site of action that is unique for each [22, 37]. The findings are nevertheless consistent with what has been observed previously with classical L-type Ca^{2+} channel blockers such as nifedipine, diltiazem, and verapamil [22, 38]. Interestingly, these selective L-type Ca^{2+} channel activators produced an oscillatory contractile response in the aorta, which is similar to what has been previously reported by Auguet *et al*., (1988). It also appeared that hibiscus and garcinia acid were more effective at relaxing the contractions induced by the Ca^{2+} channel activators compared to those induced by KCl. One possible reason could be that in these studies, KCl was applied in a hyperosmotic manner and it has been shown that a component of this contraction is independent of extracellular Ca^{2+} [39] and is somewhat insensitive to Ca^{2+} channel blockers. This may also explain why neither hibiscus nor garcinia acid caused complete relaxation of the aorta when it was pre-contracted with KCl. With regard to removal of the endothelium affecting the sensitivity of the aorta to hibiscus or garcinia acid, such an effect has also previously been observed with classical Ca^{2+} channel blockers [40] and this has been attributed to their ability to stimulate the endothelial cells to release nitric oxide [41, 42].

The contraction induced by PE involves both Ca^{2+} release from the sarcoplasmic reticulum (SR) and Ca^{2+} influx from the extracellular medium via receptor-operated (ROCCs), and VDCCs [43, 44]. Given that neither hibiscus nor garcinia acid affected the phasic contraction

produced by PE in the absence of extracellular Ca^{2+}, it seems unlikely that they are affecting Ca^{2+} release from the SR. The fact that this initial transient contraction was unaffected by either hibiscus or garcinia acid also indicates that these agents are unlikely to be affecting the biochemical sequence of events that links the increase in Ca^{2+} concentration to contraction [45]. Nevertheless, this is in contrast to what has been reported previously for the crude aqueous extract of *H. sabdariffa*, where there was ~30% reduction in the magnitude of the phasic contraction to noradrenaline [27]. Following the phasic response to PE, the re-addition of Ca^{2+} produced a sustained contraction that is due to the influx of extracellular Ca^{2+}. Both hibiscus and garcinia were found to produce relaxation of this tonic component, thereby further supporting their inhibitory effect upon Ca^{2+} influx.

While the above findings suggest that hibiscus and garcinia acid are producing vasorelaxation through inhibition of VDCCs, it is also possible that activation of K^+ channels may be involved [46]. However, the finding that the non-selective K^+ channel blocker TEA, did not prevent the relaxation to either hibiscus or garcinia acid would argue against them having a major role. There was a slight, but significant rightward shift in the concentration-response curve to both hibiscus and garcinia acid in the presence of TEA, which reflects a decrease in the sensitivity of the aorta to the relaxant effects of both acids. This could be attributed to the noted additional contraction produced by TEA itself. The decrease in potassium conductance caused by TEA, will result in depolarisation of the smooth muscle cell membrane, thereby producing a contraction [47]. Typically when K^+ channels are responsible for a relaxant effect, TEA has been found to reduce the magnitude of the relaxation by >60% [48, 49].

A variety of bioactive constituents have previously been identified in the calyces of *H. sabdariffa*, including phenolic acids, anthocyanins, and flavonoids [3]. However, very few

studies have focussed on the pharmacological activities of the constituents identified. Hibiscus and garcinia acids are diastereomeric γ-lactones derived from (2S, 3R) and (2S, 3S)-2-hydroxycitric acid, respectively [50, 51]. Only certain species of plants are known to be capable of synthesising hydroxycitric acid [52]; thus, hibiscus and garcinia acids are substances which are not found extensively in all plants. Hydroxycitric acid with the absolute configuration (2S, 3S), is a major acid component in the fruit rinds of garcinia species, including *G. cambogia*, *G. indica*, *G. Cowa,* and *G. atroviridis* [53-56]. Whereas, hydroxycitric acid with the configuration (2S, 3R) is the hibiscus-type enantiomer, which is found predominantly in the calyces of *H. sabdariffa, H. cannabinus* and *H. rosa-sinensis* [53, 57, 58]. At present, details of the of the biosynthetic pathway for hydroxycitric acid and how it is regulated remain unclear [58]. One hypothesis is that hydroxycitric acid is generated via a condensation reaction of oxaloacetate with glycolyl-CoA [58].

Both hibiscus and garcinia acids appear to have similar activity and potency with regard to their ability to cause vasorelaxation. This is somewhat unusual, since the majority of enantiomers often have unequal pharmacological properties. For example, S(-)-verapamil is more potent than the R(+)-verapamil enantiomer in terms of its vasodilatory effect, whilst diltiazem is diastereomer with two pair of enantiomers, but only the (2S, 3S) form is pharmacologically active [59]. Although rarer, there are nevertheless enantiomers that do have equal pharmacological activities; for example the antiarrhythmic flecainide and the antidepressant fluoxetine [59, 60].

6. Conclusion

This is the first report of a vasorelaxant effect for hibiscus acid, which could potentially explain the previously described vascular effects of *H. sabdariffa*, including its

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antihypertensive properties [17, 18]. This study has also shown a comparable vasorelaxant activity with the diastereomer, garcinia acid. Both acids appear to produce their vasorelaxant activity via blockade of the VDCCs in vascular smooth muscle cells, thereby inhibiting the influx of extracellular Ca^{2+}. Furthermore, this study provides support for the traditional use of the extracts or teas of *H. sabdariffa* as an antihypertensive natural product.

Conflict of interest: None of the co-authors have any conflicts of interest, including financial interests and relationships and affiliations relevant to the subject matter or materials discussed in this manuscript.

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