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Major flavonoids from *Psiadia punctulata* produce vasodilation via activation of endothelial dependent NO signaling

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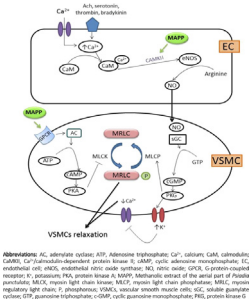
HIGHLIGHTS

- Methanol extract of *Psiadia punctulata* (MAPP) produced a significant vasodilation.
- Chloroform fraction and its methylated flavonoids were responsible for this effect.
- Vasodilation is referred to endothelial nitric oxide and, Ca^{2+} dependent eNOS.
- Interference with calcium entrance is another possible mechanism of vasodilation.

GRAPHICAL ABSTRACT



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ABSTRACT

Vasodilators are important pharmacologic agents for managing and/or treating hypertension. Medicinal plants are considered as valuable source of bioactive compounds. We used a bioguided approach to isolate, identify, and investigate the possible vasodilation activities and mechanism(s) of the prepared methanol extract from aerial parts of *Psiadia punctulata* (MAPP), its bioactive fraction and active compounds. Vascular effects of MAPP were studied using isolated artery technique in the presence or absence of specific candidate pathways inhibitors, and found to produce a significant vasodilation of phenylephrine preconstricted rat aortae. The bioactive chloroform fraction yielded five methoxylated flavonoids: umuhengerin (**1**), gardenin A (**2**), gardenin B (**3**), luteolin-3′,4′ -dimethyl ether (**4**), and 5,3′-dihydroxy-6,7,4′,5′-tetramethoxyflavone (**5**). Metabolites **1**, **4,** and **5** produced a significant vasodilation. Removal of the endothelium significantly inhibited MAPP vasodilation. Nitric oxide synthase inhibition and not prostacycline inhibition or K^+ channel blocking, was found to cause the observed vasodilation inhibition. Both guanylate cyclase and adenylate cyclase inhibitions markedly inhibited MAPP vasodilation. In conclusion MAPP possesses vasodilation activities that is mediated through endothelial nitric oxide pathway,

Abbreviations: AC, adenylate cyclase; Ca^{2+}, calcium; CaM, calmodulin; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; eNOS, endothelial nitric oxide synthase; MAPP, methanol extract from aerial parts of *Psiadia punctulata*; MDL, cis-N-(2-Phenylcyclopentyl)azacyclotridec-1-en-2-amine.HCl (MDL-12, 330A); NO, nitric oxide; NOS, nitric oxide synthase; L-NAME, Nω-nitro-L-arginine methyl ester; ODQ, 1*H*-(1,2,4)-oxadiazolo(4,3-a)quinoxalin-1-one; PE, phenylephrine; PI3K, phosphoinositide 3-kinase; PP, *Psiadia punctulata*; TEA, tetraethylammonium chloride; VSMCs, vascular smooth muscle cells; GTP, guanosine triphosphate; cGMP, cyclic guanosine monophosphate; PKG, protein kinase G.

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calcium dependent endothelial nitric oxide synthase activation, and interference with the depolarization process through calcium channel blocking activity.

Introduction

Hypertension is a leading cause of death worldwide. Although several drugs are available for treating hypertension, not all patients respond appropriately to these treatments [1]. Vascular endothelial dysfunction is characterized by lack of endothelial relaxing factors (such as, nitric oxide (NO) and H_2S), and regular vascular tone is a major risk factor for developing hypertension [2]. There is a growing interest in bioactive compounds from plant sources that could be used to treat hypertension. About two hundred metabolites from plants belong to different classes of phytochemicals have been examined for their vasodilator activity [3]. These compounds include flavonoids (Luteolin, quercetin, kaempferol, epicatechin and naringin), sesquiterpene (polygodial), monoterpene (rotundifolone), and alkaloid (rutaecarpine) [3,4]. Flavonoids with cardiovascular protective effect are potentially useful for treating or reducing the progression of cardiovascular diseases, like hypertension [5]. They show various mechanisms of action that include increasing NO bioavailability, reducing oxidative stress, inhibition of protein kinase C, inhibition of cyclic nucleotide phosphodiesterases, and/or acting on vascular ion channel activity to decrease calcium uptake [3,5,6].

Psiadia punctulata (PP) is a small shrub found mostly in the tropics of Asia and Africa, as well as in Saudi Arabia [7]. The plant is characterized by the presence of different classes of phytochemicals, including diterpenes, flavonoids, and phenylpropanoids [8–10]. PP has traditionally been used to treat cold, abdominal pain, fever, malaria, scabies and skin infection; as analgesic and expectorant; and to remove ectoparasite from cattle [8,9]. Other studies have reported PP leaves to exhibit antifungal and pesticide activities [11], while its bark showed antiprotozoal activity against *Leishmania major* [12]. A 70% methanolic extract of the whole plant is also reported to show a blood pressure lowering effect, phrenic neuromuscular nerve blocking effect, and relaxant properties on unstriated muscle of guinea pig trachea [13]. Furthermore, methanol extract of PP leaves was demonstrated to inhibit Ca^{2+} ions induced rabbit jejunum constrictions which suggests calcium channel blocking activity [13]. It was suggested that this Ca^{2+} channel blocking activity may be responsible for the extract antihypertensive effect, yet the precise mechanism of action remains unknown[13]. It is also proposed that the vasodilator activity of PP on constricted vessels could be attributed to the protective effect of its flavonoids against advanced glycation end product [14]. For this reason, we decided to explore the possible vasodilator effect of MAPP by identifying the bioactive fraction, isolating the biometabolites, and elucidating the mechanism of action.

Materials and methods

Detailed procedures for plant extraction, fractionation and isolation of bioactive compounds [14] as well as HPLC quantification of isolated compounds in the bioactive chloroform fraction are available in supplementary data. Briefly, MAPP was extracted with chloroform (Fr. I), and remaining mother liquor was fractionated on polyamide column using water, methanol/water (1:1) and finally methanol to give fractions II-IV. Based on the bioactivity of the isolated fractions, Fr. I, and not Fr. II-IV was subjected to different chromatographic columns to isolate the major bioactive compounds as previously described [14].

Aortae isolation

Aortae was prepared as previously described [15–17], following the guidelines of Faculty of Pharmacy Research Ethics Committee (King Abdulaziz University, approval number PH-119-39). Briefly, rats were anesthetized with ketamine (100 mg/kg, i.p.), then part of the thoracic cage was removed and the descending thoracic aortae was carefully excised and placed in a Petri dish filled with cold Krebs–Henseleit solution. The aorta was then cleaned of connective tissues and fats, and cut into rings of approximately 3 mm long.

Studying the vasodilation effect

The vasodilation effects of MAPP, fractions and isolated compounds were studied as previously reported [15–17] using the isolated artery techniques. Briefly, isolated aortae rings were preconstricted with 1 μM of phenylephrine (PE), then cumulative concentrations of MAPP (1–32 μg/ml), different fractions (I–IV) (1–32 μg/ml), or tested metabolites (**1–5**) (1–32 μg/ml) were added to the organ baths. The changes in tension were then recorded. The concentration of PE (1 μM) is the submaximal concentration of PE used to precontract vessels before studying vasodilation as previously reported [18].

Other potential mechanisms of action were explored by testing cumulative vasodilation responses to MAPP, and the isolated bioactive compounds in endothelium denuded vessels (through luminal surface gentle rubbing; just sufficient to denude the endothelium while leaving smooth muscle function) [19] and in the presence or absence of certain pathways specific inhibitors. These include (i) atropine (100 μM) to block muscarinic receptors [20]; (ii) propranolol (1 μM) to block β-adrenergic receptors [20]; (iii) Nw-nitro-L-arginine methyl ester (L-NAME; 100 μM), a nitric oxide synthase (NOS) non-selective inhibitor [21]; (iv) indomethacin (10 μM), a cyclooxygenase inhibitor [22]; (v) high extracellular K^+ (KCl, 30 mM) to prevent hyperpolarization of membrane [20]; (vi) tetraethylammonium chloride (TEA, 10 mM) to block the Ca^{2+}-activated potassium channels [23]; (vii) *cis*-N-(2-Phenylcyclopentyl)azacyclotridec-1-en-2-amine.HCl (MDL-12, 330A, MDL, 30 μM), an adenylate cyclase (AC) inhibitor [24]; (viii) 1*H*-(1,2,4)-oxadiazolo(4,3-a)quinoxalin-1-one, (ODQ, 10 μM), a guanylate cyclase inhibitor [25]; (ix) wortmannin (0.1 μM), a phosphoinositide 3-kinase (PI3K) inhibitor [26]; and (x) KN-93 (10 μM), a Ca^{2+}/calmodulin dependent protein kinase II (CaMKII) inhibitor [27].

The effect of extracellular Ca^{2+} on the vasodilation produced by MAPP was investigated as detailed in our previous publication [28]. In brief, after 30 min stabilization period in normal Krebs solution, the physiological buffer was changed into Ca^{2+} free Krebs buffer and kept for another ten minutes. KCl (80 mM) was then added and the response was reordered. Following, responses after raising $CaCl_2$ concentrations (1.25 to 5 mM) were also recorded in absence or presence of fraction I (3–30 μg/ml) or the vehicle (control group; 0.1% dimethyl sulfoxide).

Drugs and chemicals

Propranolol, MDL, ODQ, wartmannin, KN-93 (LKT Laboratories, Inc.Paul, Minnesota, USA), PE, TEA, L-NAME, atropine, indomethacin, and dimethyl sulfoxide (Sigma Aldrich, Munich, Germany)

were used in this study to evaluate the antihypertensive effect of MAPP.

Statistical analysis

The analysis was carried through Graph pad Prism 5.0 (Graph Pad, USA) and the results were represented as mean ± SEM. Statistical comparison was done using two-way ANOVA and Bonferroni post-hoc tests, respectively and the $P < 0.05$ value was taken into consideration as significant.

Results

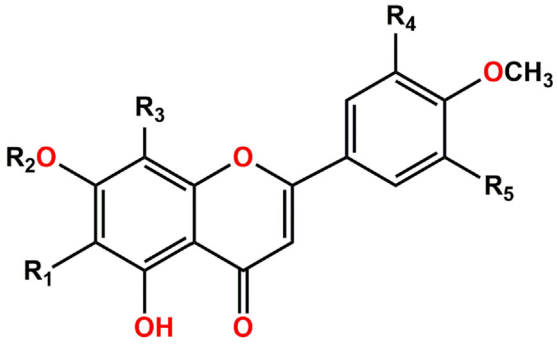
As described below, MAPP, the various fractions and isolated methoxylated flavonoids were tested for their vasodilator activity using isolated artery technique. Among the four fractions, only the chloroform fraction (Fr. I) showed significant vasorlexant activity, and was found to contain five major methoxylated flavonoids (**1–5**) in concentrations of 55.1, 6.3, 66.3, 65.5, 73.2 mg/g, respectively as determined by HPLC (supplementary data), with the metabolites **1**, **4,** and **5** producing the most significant vasodilation activities.

Identification of isolated compounds

Further investigation of the PP chloroform fraction yielded five major methoxylated flavonoids: umuhengerin (**1**), gardenin A (**2**), gardenin B (**3**), luteolin-3′,4′-dimethyl ether (**4**), and 5,3′-dihy droxy-6,7,4′,5′-tetramethoxyflavone (**5**) [14] that were characterized utilizing NMR techniques (supplementary data) (Fig. 1). These flavonoids represented the major constituents of chloroform fraction as concluded from HPLC analysis (supplementary data).

The vasodilating effect of MAPP and fractions

Fig. 2A shows cumulative relaxation response curves to MAPP, and its fractions (I–IV), where MAPP produced significant vasodilation in aortae vessels at concentrations of 10 and 32 μg/ml. Similar effect was observed for fraction I as it produced significant vasodilation in aortae vessels at concentrations 3, 10 and 32 μg/ml. On the other hand, other fractions II, and III did not possess any relaxant activity on aortae vessels, while fraction IV resulted in only weak vasodilation, and only at the highest concentration of 32 μg/ml (Fig. 2A). Based on these observation, the non-chloroform fractions (Fr. II-IV) were not studied further.



	R_1	R_2	R_3	R_4	R_5
1	OCH_3	CH_3	H	OCH_3	OCH_3
2	OCH_3	CH_3	OCH_3	OCH_3	OCH_3
3	OCH_3	CH_3	OCH_3	H	H
4	H	H	H	OCH_3	H
5	OCH_3	CH_3	H	OH	OCH_3

Fig. 1. Chemical structures of isolated compounds from *P. punctulata.*

The vasodilating effect of isolated compounds from fraction I

Fig. 2B shows that compound **5** produced the most relaxation effect in aortae vessels at concentrations 10 and 32 μM, followed by compounds **1** and **4**, while compounds **3** and **2** did not show any significant activity at all concentrations.

Role of endothelium in the observed vasodilation

Since fraction I caused the largest vasorelaxation with similar effect as MAPP, further detailed mechanistic studies with this fraction were performed with aortae. The result showed vasodilation produced by fraction I was significantly attenuated by endothelial denudation at 10 and 30 μg/ml (Fig. 3A).

Receptors involved in the observed vasodilation

In attempt to identify the possible receptor(s) implicated in fraction I observed vasodilation activity, we tested fraction I in the presence of propranolol (β-adrenergic receptor antagonist) and atropine (muscarinic receptor blocker). The vasodilation property of fraction I was significantly attenuated by propranolol (Fig. 3B). Atropine did not show any significant effect on fraction I vasodilation activity.

Major pathways involved in PP vasodilation

In search for the major pathways involved in fraction I vasodilation, we tested the activity of fraction I in the presence of L-NAME (NO synthase inhibitor), which significantly inhibited fraction I vasodilation at all concentrations (Fig. 3C). However, none of the prostaglandin synthase inhibitor (Indomethacin), standard voltage dependent K^+ channel blocker (TEA), indomethacin, or membrane hyperpolarization by KCl showed any significant effect on fraction I vasodilation (Fig. 3C).

Mechanisms underlying PP vasodilation

The role of cyclases in MAPP vasodilation was also studied. Both guanylate cyclase inhibitor, ODQ and AC inhibitor, MDL significantly inhibited fraction I vasodilation activity (Fig. 4A). Similarly, CaMK inhibitor, KN-93 also inhibited fraction I vasodilation activity (Fig. 4B). However, PI3K inhibitor, wartmanin did not have any significant effect on fraction I vasodilation.

Inhibition of Ca^{2+} influx and mobilization seem to play a role in PP vasodilation

Incubation of fraction I (3, 10, and 30 μg/ml) with aortae sections preconstricted by KCl in Ca^{2+}-free media inhibited Ca^{2+} induced constriction of aortae vessels (Fig. 5).

*Major mechanisms contributing in vasodilation effect of compounds **1**, **4**, and **5**.*

Relaxation effects of compounds **1** and **5** were significantly attenuated by endothelial denudation at concentration 10 and

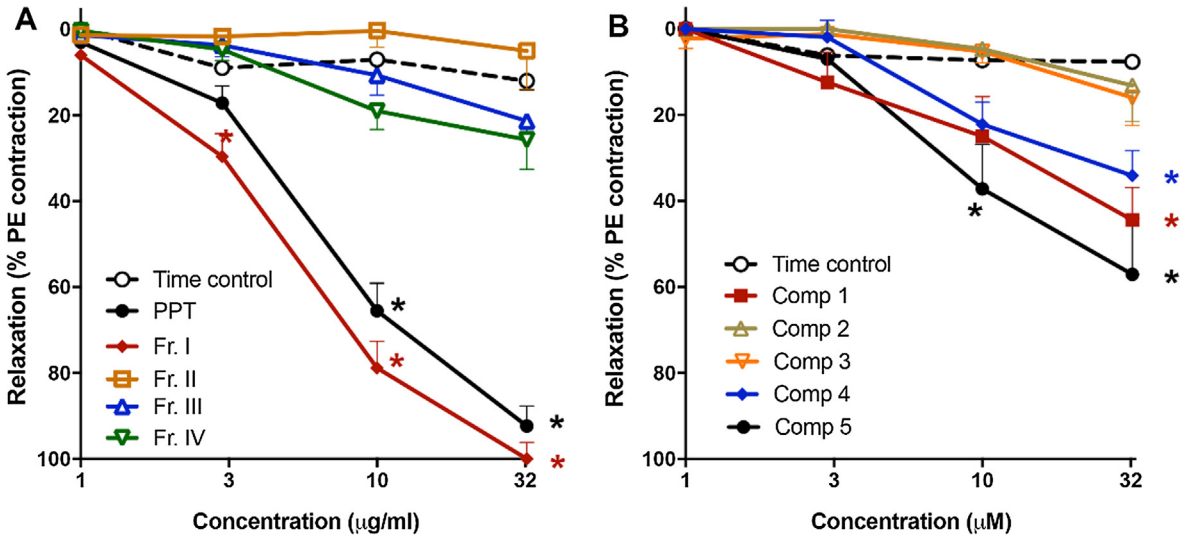


Fig. 2. Effect of MAPP on vascular relaxation. (A) Cumulative relaxation-response curves to MAPP, fractions I- IV in rat aortae, compared with appropriate time controls, (B) Cumulative relaxation-response curves to isolated compounds from fraction I in rat aortae, compared with appropriate time controls. Relaxations are expressed as a percentage of the initial PE-induced constriction. Data are presented as mean ± standard error of 6 animals. *P < 0.05, compared with the time control values; by two Way ANOVA and Bonferroni post hoc test.

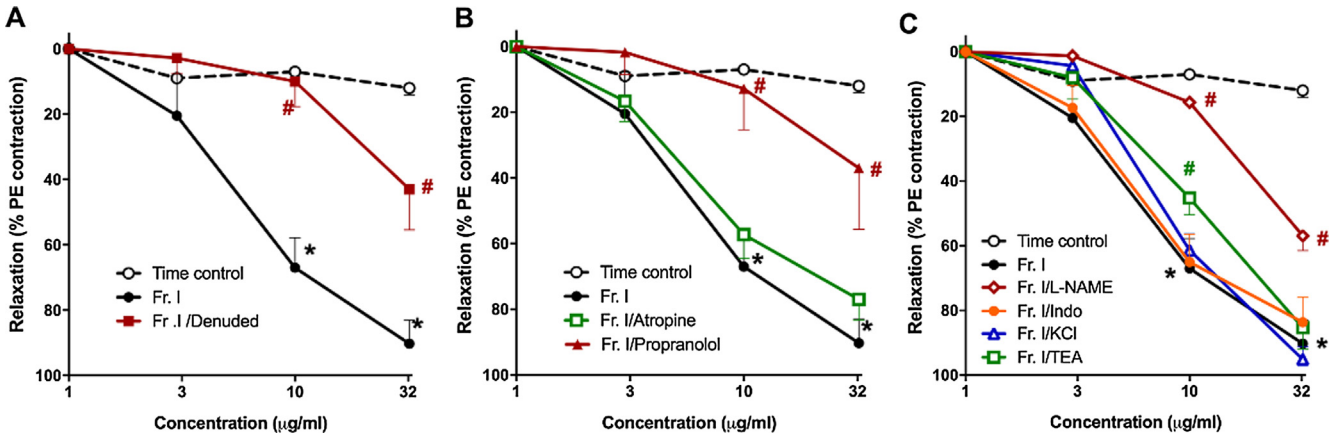


Fig. 3. Effect of *in vitro* addition of cumulative concentrations of fraction I (1–32 μg/ml) on phenylephrine (μM)-preconstricted isolated aortae. The effect of (A) denudation, (B) preincubation (20 min) with a β-adrenergic receptor antagonist, propranolol and the standard muscarinic receptor blocker, atropine, and (C) preincubation (20 min) with the nitric oxide synthase inhibitor Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME, 100 μM), the cyclooxygenase inhibitor indomethacin (INDO, 5 μM), membrane hyperpolarizing agent KCl, and the standard voltage dependent K^+ channel blocker, tetraethylammonium chloride (TEA) on the vasodilation effect of fraction I on phenylephrine (PE) preconstricted aortae. Data are presented as mean ± standard error of 6 animals. *P < 0.05, compared with the time control values, #P < 0.05, compared with PP fraction I values; by two Way ANOVA and Bonferroni post hoc test.

32 μM for compound **5** and concentrations of 3, 10, and 32 μM for compound **1** (Fig. 6). Similarly, relaxation effects of compounds **1** and **5** were diminished by L-NAME or ODQ at concentrations 10 and 32 μM (Fig. 6).

Relaxation effect of compound **4** was also attenuated by endothelial denudation and by ODQ at concentrations 10 and 32 μM. However, the NOS inhibitor, L-NAME had no significant effect on compound **4**-induced relaxation (Fig. 6).

Discussion

The current study reports the vasodilation effect of MAPP, and its bioactive compounds using isolated aortae vessels. The chloroform fraction (Fr. I) is found to be responsible for the observed vasodilation activity of MAPP. Among the five isolated compounds (**1–5**) from fraction I: only **1**, **4**, and **5** possess vasodilation activities on isolated rat aortae sections. The vasodilation activity appears to be mediated by specific activation of endothelial dependent NO signaling. We note that the non-chloroform fractions (Fr. II-IV) did not result in any significant vasodilatory activity, and thus were not studied further.

In search for the possible mechanism of the MAPP vasodilation activity, several experiments were conducted using Fr. I and the isolated bioactive compounds **1–5**. Mechanical removal of the endothelium blocked the vasodilation induced by Fr I. We also showed that inhibiting endothelial NO generation by L-NAME inhibited the MAPP vasodilation activity. Meanwhile; prostaglandin inhibition by indomethacin did not show any effect on the observed vasodilation activity. This proves that Fr I induces vasodilation through endothelium dependent NO mechanism. The critical role of endothelium in vasodilation through NO generation and prostacycline synthesis has been widely accepted [29].

To determine the possible receptor(s) involved in the observed vasodilation of MAPP, we performed a series of experiments using atropine and propranolol. The results indicate that Fr I exerts its

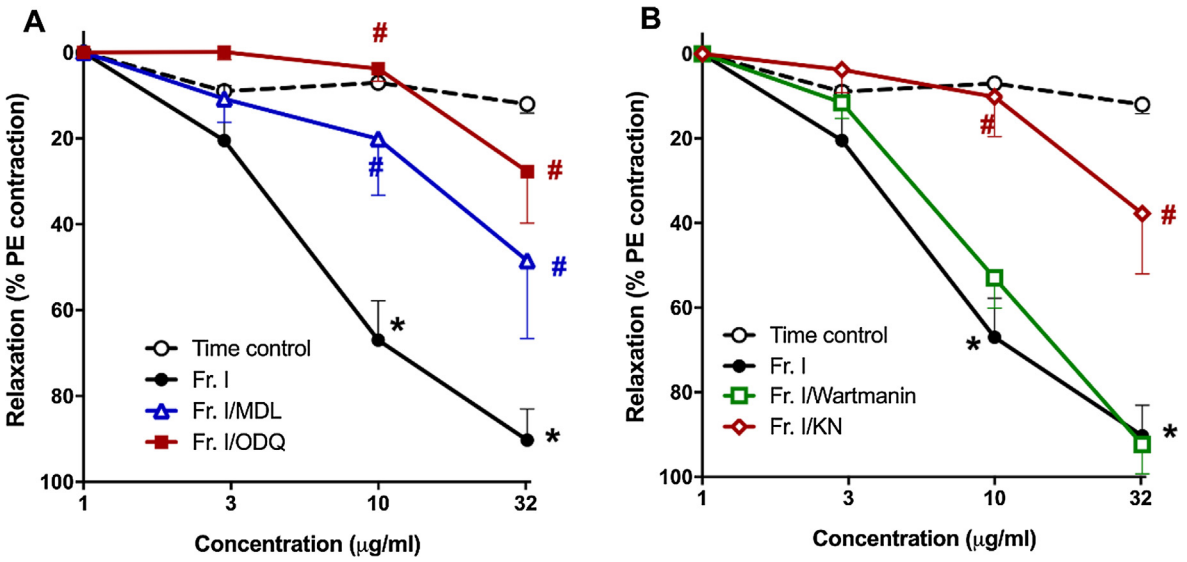


Fig. 4. Effect of *in-vitro* addition of cumulative concentrations of fraction I (1–32 μg/ml) on phenylephrine (μM)-preconstricted isolated aortae. The effect of preincubation (20 min) with (A) the guanylate cyclase inhibitor, ODQ, adenylate cyclase inhibitor, MDL, (B) The phosphoinositide-3-kinase inhibitor, wartmanin and the Ca^{2+}/calmodulin-dependent protein kinase inhibitor, KN-93 on the vasodilation effect of fraction I on phenylephrine (PE) preconstricted aortae. Data are presented as mean ± standard error of 6 animals. *P < 0.05, compared with the time control values, #P < 0.05, compared with fraction I values; by two Way ANOVA and Bonferroni post hoc test.

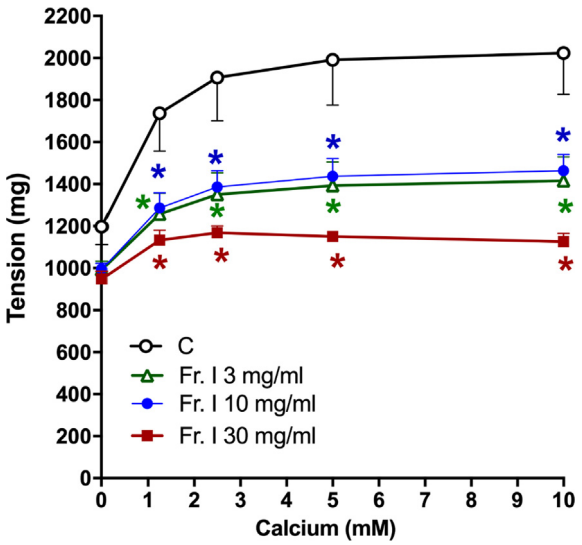


Fig. 5. Concentration-response curve of $CaCl_2$ on KCl (80 mM)-induced constriction of aortic rings in the absence (control) or presence of different concentration of fraction I (3, 10, and 30 μg/ml) in a Ca^{2+} free Krebs solution.

actions in the endothelium via binding to β-adrenergic receptor. As β-adrenergic receptor is coupled to G-protein–AC system [30], we utilized the AC inhibitor, MDL, which significantly inhibited the observed vasodilation, strongly suggesting activation of AC to play a role in MAPP vasodilation activity.

Vasodilation mediated by NO is mainly through cytosolic soluble guanylate cyclase activation in smooth muscle followed by cyclic guanosine monophosphate (cGMP) dependent protein kinase G (PKG) activation [31]. This observation is consistent with our study with the selective guanylate cyclase inhibitor ODQ, which significantly inhibited MAPP vasodilation activity. Endothelial nitric oxide synthase (eNOS) is a significant regulatory enzyme that catalyze the production of NO from arginine [32], while CaMKII is proposed to transduce the downstream effects of Ca^{2+}/calmodulin (CAM) [33]. CaMKII is also suggested to be involved in NO synthesis through Ca^{2+}-dependent activation of eNOS [34]. Our results demonstrated that CaMKII inhibitor, KN-93 attenuated aortae vessels dilatation to MAPP, suggesting that MAPP induced production of NO involves CaMKII dependent activation of eNOS. Taken together, the current results highlight the important role of NO in MAPP induced vasodilation. The study also point to Ca^{2+} dependent activation of eNOS and subsequent PKG cGMP dependent activation.

Calcium, together with CAM is a key player in mediating smooth muscle constriction [35]. Membrane depolarization is a fundamental regulator of ion channels in smooth muscle cells especially voltage dependent Ca^{2+} channels [36]. Inhibitory effects on vascular calcium currents have been described for several flavonoids [37]. The isoflavone genistein was reported to inhibit vascular Ca^{2+} currents in myocytes isolated from rabbit ear arteries in a dose-dependent manner [38]. Furthermore, the flavonoid scutellarin has been shown to relaxe both endothelium intact and endothelium denuded rat aortic rings due to inhibition of calcium influx [39].

In this study, we assessed the possible role of Ca^{2+} on MAPP vasodilation effect. Ca^{2+} induced concentration dependent constrictions in the isolated aortae preconstricted with KCl, which was attenuated by incubation of aortae sections with different concentrations of MAPP. Calcium channel blocking activities has been previously reported in rabbit jejunum for methanol extract of leaves of PP [13].

Flavonoids are ideal source for antihypertensive agents [40], with examples like luteolin, quercetin, rutin, kaempferol, rhoifolin, and apigenin acting as angiotensin converting enzyme inhibitors [40]. Structure activity relationship (SAR) of flavonoids as vasodilators was examined previously based on their mechanism of increasing NO bioavailability and calcium channel blocking activity [41,42]. A previous study had correlated antihypertensive activity of flavonoids, with the presence of four hydroxyl/methoxyl groups in these compounds [37]. It was reported that vasodilation by NO formation requires the presence of flavan moiety with free hydroxyl residues at C-3, C-3′, C-4′, C-5 and C-7 and a hydroxyl-, oxo-, or phenolic substituent at C-4 [41]. Regarding calcium channel blocking activity of flavonoids, it was reported that flavones (luteolin) and flavonols (quercetin) showed the highest activity [6,42].

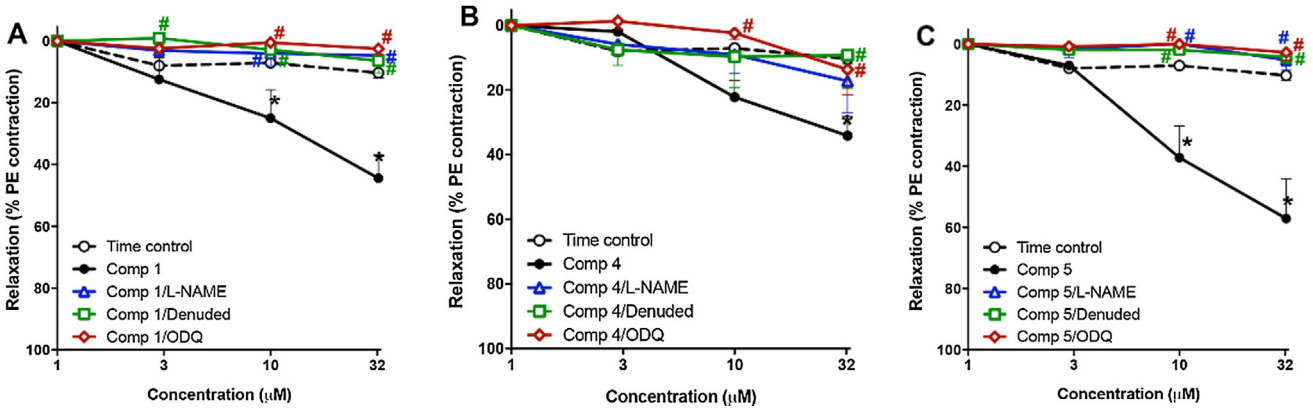


Fig. 6. The vasodilation effect of *in-vitro* addition of cumulative concentrations of isolated compounds from PP fraction I: (A) compound **1**, (B) compound **4** and (C) compound **5** (1–32 μM) on phenylephrine (μM)-preconstricted isolated aortae which is either denudated or preincubated (20 min) with the nitric oxide synthase inhibitor Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME, 100 μM) or the guanylate cyclase inhibitor, ODQ. Data are presented as mean ± standard error of 6 animals. *P < 0.05, compared with the time control values, #P < 0.05, compared with compounds **1** or **4** or **5** values; by two Way ANOVA and Bonferroni post hoc test.

However, the exact structural features for its vasodilator activity is not yet established. Available literature agree on the importance of ketonic group at C-4 for vasodilation activity [42]. The presence of double bond between C-2 and C-3 has also been suggested to result in strong vasodilation activity [42], however, other reports suggest that their absence (hesperetin and 6-hydroxy flavanone) did not affect calcium channel blocking activity [43]. Calcium induced contraction in rat isolated thoracic aortae has been suggested to be impaired by different flavonoids, meanwhile, 3′,4′-dimethoxy flavonol, and flavones are less active [44]. Moreover, glycosylation has been reported to greatly abolish the vasodilating effect [6,42]. The above reports are controversial with those obtained by Calderone et al 2004 regarding methoxylated flavonoids. He reported a strong vasorelaxant effect of acacetin (methoxylated flavonoid) due to its calcium channel blocking activity [43]. Moreover; it was shown that pentamethoxyflavone produced relaxation of thoracic aortic rings by stimulating the release of nitric oxide and H_2S, that act as an adenylyl cyclase stimulator, and an inhibitor of intracellular calcium mobilization [45]. In addition, 3,5,7,4′-tetrahydroxy-8-methoxyflavone showed vasorelaxation similar to that of quercetin in isolated rat aortae contracted with high KCl or with noradrenaline [46].

In our study, compounds **1**, **4** and **5** that showed the highest activities contain five, two and four methyl ether groups, respectively. Meanwhile, compounds **2** and **3** with six and four methyl ether groups showed the least activity. Based on these observations, it could be concluded that structure features for vasodilator flavonoid include; a flavan nucleus with a ketonic group at C-4, and methoxylated groups in rings A and B where the presence of high number of oxygenated carbons showed higher activity (compounds **1** and **5**). These results require further investigation of antihypertensive activity of methoxy flavonoids to explore their structure activity relationship as the number of methyl ether groups may not be the only factor that affects their activity, especially with the low activities of compounds **2** and **3**.

Conclusion

In conclusion, MAPP, chloroform fraction as well as its methoxylated flavonoids exert significant vasodilation effects via activation of a specific endothelial NO signaling cascade and through Ca^{2+} channel blocking activity. This indicates that PP may be beneficial in disease states that are associated with cardiovascular complications. This study presents MAPP and its methoxylated flavonoids as attractive therapeutic option worthy of further investigation. It is obvious that antihypertensive activity of the plant is mainly related to its methoxylated flavonoid contents which represent the major compounds in chloroform fraction as concluded from HPLC analysis. Meanwhile, other compounds may be present in the bioactive fraction in lower concentrations, and possibly show vasodilator activity, which will be an opportunity for further investigations by researchers to identify these additional bioactive compounds.

Compliance with Ethics requirements

All Institutional and National Guidelines for the care and use of animals (fisheries) were followed.

Declaration of Competing Interest

The authors declared that there is no conflict of interest.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jare.2020.01.002.

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