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Possible Mechanism of Action of the Hypotensive Effect of *Peperomia pellucida* and Interactions between Human Cytochrome P450 Enzymes

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Abstract

Background: Peperomia pellucida is used as a medicinal plant and as an antihypertensive remedy. We investigated the possible mechanism of this action and its impact on cytochrome P450 (CYP) enzyme activity.

Methods: Mean arterial pressure and heart rate were recorded via cannulation of the carotid artery on anaesthetized, normotensive Sprague-Dawley rats following intravenous administration of Peperomia pellucida aqueous (10-30 mg/kg) plant extract (PPAE). Recordings of the contractile activity of the aortic rings to the extract (1.9-8.6 mg/ml) were done using standard organ bath techniques. Impact on CYP3A4 and CYP2D6 enzyme activities was investigated using human liver and heterologously expressed microsomes.

Results: We observed a dose-dependent reduction in systolic, diastolic, MAP and HR. Pre-treatment with atropine (2 mg/kg) and propranolol (1 mg/kg) but not mepyramine (2 mg/kg) significantly ($p<0.05$) reduced the hypotensive and negative chronotropic activities caused by the extract, while L-NAME (5 mg/kg) completely abolished it. PPAE significantly ($p<0.05$) relaxed the phenylephrine (10^{-9}-10^{-4} M) and KCl- induced contractions and displayed moderate inhibition of CYP3A4 enzyme activity with IC_{50} values of 0.466 ± 0.126 mg/mL and 0.153 ± 0.054 mg/mL, respectively using heterogenously expressed CYP3A4 and human liver microsomes (HLMs)

Conclusion: Results suggest dose-dependent hypotensive, bradycardic and vasorelaxant effects of PPAE are mediated through Nitric oxide-dependent mechanisms. The impact on CYPs enzyme activities indicate unlikely adverse drug effect when Peperomia pellucida is consumed with other medications reliant on CYP3A4 metabolism.

Keywords: Hypertension; Endothelium; Peperomia pellucid; Cytochrome P450 (CYP450); Mechanism of action

Background

Hypertension is a major cardiovascular burden that has been estimated to cause 7.1 million premature deaths and 45 % of the disease burden [1]. However, it remains inadequately managed everywhere [2], and in spite of the large number of antihypertensive medications, most people in developing countries have poor access to modern health-care and cannot afford these drugs due to cost. Therefore, they resort to alternative herbal remedies to manage hypertension.Such alternative remeadies include the use of herbs and natural plant products, one of the plant used is Peperomia pellucid [3]. *Peperomia pellucida* is a common, fleshy annual herb that belongs to the family of Piperaceae. It is commonly called shiny bush, pepper elder, man-to-man, rat-ear, *Pansit pansitan* and is found mainly in the tropics [4-6].

Traditionally, the plant is used as a diuretic and to reduce cholesterol levels in the treatment of hypertension and kidney disorder [4-6]. It is also reported to have anti-inflammatory properties [6] analgesic activity[4,6-8] antipyretic [9] as well as antibacterial [5,7,10] also used in the treatment of abscesses, furuncles, and conjunctivitis [6].

The phytochemicals present in the plant are alkaloids, namely, secolignans, tetrahydrofuran lignans, as well as, highly methoxylated dihydronaphthalenone, peperomins A, B, C, and E, sesamin, and isoswertisin [11]. Peperomin E shows growth inhibitory effects on the HL-60, MCF-7, and HeLa three cancer cell lines [7,11]. *Peperomia pellucida* also contains several essential oils, mainly dillapiole, β-caryophyllene and carotol that have high larvicidal activities [12-14]. Other compounds are flavonoids such as acacetin, apigenin, isovitexin and pellucidatin [15,16], phytosterols, namely, campesterol, stigmasterol, and arylproppanoids. Cardiac glycosides, tannins and anthraquinones have also been isolated from the plant [4].

Given the widespread ethno medicinal use of this plant and its usage in combination with other medications, it was important to investigate impact on drug metabolising cytochrome P450s (CYPs), a heme containing superfamily of enzymes [17], Herbal remedies are known to affect the dynamics of drug and chemical interactions [18], and of significant concern to drug-drug interactions [19,20]. There is therefore need for pharmacological validation of this medicinal plant to justify its usage and safety in ethno medicinal treatment, which could greatly benefit populations with poor economic resources. Therefore, the aim of the present study was to investigate the blood pressure lowering effects and possible mechanism of action of an aqueous extract of *Peperomia pellucida*, and its possible drug-herb interactions using CYP540 microsomes.

Methods

Chemicals and reagents

All chemicals except those noted below were purchased from

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Sigma-Aldrich (St. Louis, MO). All CYP substrates and metabolites were purchased from Gentest Corporation (Worburn, MA, USA). *Escherichia coli* membranes expressing human CYP2D6 and CYP3A4 co-expressed with CYP reductase were purchased from Cypex Ltd. (Dundee, UK), while pooled human liver microsomes were purchased from Xenotech (Kansas, USA).

Plant material and extraction

The *Peperomia pellucida* whole plant (1 kg) was collected in January 2010 and the species authenticated by the resident botanist (Mr Patrick Lewis) at the herbarium of the University of The West Indies (UWI) where a voucher specimen of the plant material has been deposited (35447). The whole plant was washed, dried, ground into powder and soaked in distilled water overnight. It was filtered using Whatman No. 1 filter paper and the filtrate was concentrated under reduced pressure at 45°C in a rotary vacuum evaporator. The dark brown solid residue was stored in a capped container in a refrigerator at -4°C until ready for use. All the drugs used were dissolved in distilled water prior to use. All solutions were freshly prepared prior to the start of experimental procedures.

Experimental animals

Male Sprague Dawley rats, aged 12 weeks and weighing 300-350 g were obtained from the Animal House of the Department of Basic Medical Sciences, UWI, Mona Campus. They were housed in plastic cages under 12 h light/dark cycles at 25 ± 2°C and fed with standard rat chow and tap water *ad libitum*. Ethical approval was sought and obtained from the FMS/UHWI/UWI, Mona Campus Ethics committee.

Measurement of blood pressure and heart rate

The animals were anaesthetized with an intraperitoneal injection of 15% urethane (8 ml/kg body weight). The trachea was exposed and cannulated to facilitate easy respiration. The left jugular vein was cannulated to facilitate the intravenous injection of the drugs and plant extracts. The right carotid artery was cannulated and connected to a pressure transducer (Statham P23 XL) coupled with a Grass Polygraph (Model 7D, Quincy, MA, USA). This connection was used for blood pressure and heart rate recording. 500 IU/kg of heparin (Elkins-Sinn Inc., Cherry Hill NJ., USA) was injected to prevent intravascular blood clotting. The animals were allowed to adapt to the laboratory setting for at least 30 min before recording and administration of any test substances. Drugs and the aqueous plant extract were then administered intravenously.

Dose response effects of *Peperomia pellucida* extract on blood pressure and heart rate

After the equilibration period, the dose-response relationship to *Peperomia pellucida* extract was determined by intravenous injection (10-30 mg/kg) into the left jugular vein and flushed in with 0.1 ml saline. Each dose was separated by 10 min interval before the injection of the next dose. The blood pressure was recorded at a chart speed of 10 mm/s and the heart rate was measured by increasing the chart speed on the machine to 50 mm/min. The mean arterial pressure (MAP) was calculated as the sum of the diastolic pressure and 1/3 pulse pressure.

Effect of *Peperomia pellucida* on atropine, propranolol, mepyramine and eNOS blockade

The effect of *Peperomia pellucida* extract was examined after administration of the muscarinic receptor antagonist, atropine (2 mg/kg), the beta-adrenoceptor antagonist, propranolol (1 mg/kg), mepyramine (5 mg/kg) or N^{ω}-nitro-L-arginine methyl ester (L-NAME 5 mg/kg). Each drug was given intravenously and allowed to equilibrate for 5 min before a bolus injection of *Peperomia pellucida* extract (10 mg/kg) (which was a representative dose that gave about 40 – 50% of the effects observed) and the corresponding blood pressure and heart rate changes were recorded.

Preparation of rat aorta rings

The thoracic aorta were isolated from male rats and after removal of superficial fat and connective tissue, cut into rings of about approximately 3 mm and mounted in 10 ml organ baths containing normal Krebs physiological solution with the following composition (mM): NaCl, 112; KCl 5; CaCl2 1.8; MgCl2 1, NaHCO3 25; KH2PO4 0.5; NaH_2PO_4 0.5; Glucose 10; pH 7.4. The bath solution was maintained at 37°C and bubbled continuously with a mixture of 95% O_2 and 5% CO_2. The aorta was connected to an isometric force transducer (SS12LA, Biopac Systems Inc, Goleta, CA, USA), connected to a data acquisition unit (Biopac Student Lab MP36 systems) and isometric contraction was recorded using the Biopac BSL PRO computer software. A passive tension of 1 g was applied to the tissue using a movable device. The rings were equilibrated for 90 min while being rinsed every 15 min. During the equilibration period, the rings were challenged with 1 μM phenylephrine and the aorta was relaxed with 10 μM acetylcholine to ascertain the endothelial integrity.

Characterization of the vasorelaxant responses to *Peperomia pellucida*

After the equilibration period, the aortic rings with or without endothelium were precontracted with 1 μM phenylephrine and the relaxant responses to *Peperomia pellucida* at different concentrations (1.9-8.6 mg/ml) were recorded by adding cumulative doses of aqueous extract to the tissue bath after the previous concentration had reached a plateau. Endothelium was removed mechanically by gently rubbing the intimal surface of the aortic rings with glass rod and removal was confirmed by the absence of relaxation to 10-7 M acetylcholine [21]. In another set of experiments, aortic rings with intact endothelium were pre-incubated with *Peperomia pellucida* extract for 30 min following which cumulative dose-response curves were generated for phenylephrine. Dose-response curves were plotted as percentage relaxation against logarithmic concentration of the extract.

Characterization of vasorelaxant action

In order to determine the involvement of intracellular Ca^{2+}mobilization in the vasorelaxant action of *Peperomia pellucida*, Ca^{2+}-free Krebs solution with the following composition: KCl 50, NaCl 91.04, $MgSO_4$ 1.05, $NaHCO_3$ 11.90, glucose 5.55 and EGTA 0.1 mM was used. To confirm the calcium channel blocking effect, the tissue was allowed to stabilize in normal Kreb's solution, which was then replaced with Ca^{2+}-free Kreb's solution containing EGTA (0.1 mM) for 30 min with 4-5 serial washing in order to remove calcium from the tissues. The aortic ring was assessed by testing on high K^+ (80 mM)-induced contraction. This solution was further replaced with K^+-rich and Ca^{2+}-free Kreb's solution. Following an incubation period of 30 min, dose-response curves of Ca^{2+}were obtained and then repeated following 30 min incubation with the *Peperomia pellucida* extract.

CYP inhibition assays

The extract was evaluated for its ability to inhibit the catalytic activity of human CYP3A4 enzyme by means of fluorometric detection assays conducted in 96 well microtitre plates using the substrate;

7-Benzyloxy-4-trifluoromethylcoumarin (BFC) for detecting CYP3A4 activity as described elsewhere [22,23]. For experiments with pooled HLMs, 300 µg/ml of protein was used in each assay. The reactions were monitored fluorometrically at 37°C, using a Varian Cary Eclipse Fluorescence spectrophotometer. All standards were dissolved in 20% acetonitrile and less than 0.3% of acetonitrile was used in the final assay.

The accuracy of experimental techniques employed to detect CYP3A4 inhibition assay was verified with known inhibitor ketoconozole and the obtained IC_{50} value (0.06 ± 0.01 µM) compared well with published values (0.06 µM) [24]. Michaelis constant, Km, was determined for the marker substrate under the specified experimental conditions, in order to determine suitable substrate concentrations for assessing inhibitory potential of the test extract. Control experiments included the intrinsic fluorescence of the *Peperomia pellucida* extract and its effect on the metabolite's fluorescence at the respective excitation and emission wavelengths.

Data analysis

The results are expressed as mean ± SEM. Student's t-test and one-way analysis of variance (ANOVA) with Bonferonni's post-test was performed where applicable using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, Ca, USA). IC_{50} values were determined by fitting the data in Sigma Plot (version 10.0) and enzyme kinetics module, using non linear regression analysis. IC_{50} data listed represent the average values from three different determinations. A *p* value of 0.05 was considered statistically significant.

Results

Effect of graded doses of *Peperomia pellucida* on blood pressure and heart rates

Intravenous administration of increasing dose *Peperomia pellucida* aqueous extract caused a dose-dependent reduction in systolic blood pressure (SBP), diastolic blood pressure (DBP), Heart rate and MAP (Table 1).

Mechanism of hypotensive effect of *Peperomia pellucida*

The effects of atropine, propranolol, mepyramine and L-NAME on the hypotensive action of the aqueous extract of *Peperomia pellucida* (10 mg/kg) were investigated. As shown in Figure 1, the pretreatment of anaesthetized Sprague Dawley normotensive rats with atropine sulphate (2 mg/kg) or propranolol (1 mg/kg) significantly (p<0.05) reduced the hypotensive effect of the plant extract. However, mepyramine (5 mg/kg) caused a significant (p<0.05) further reduction of MAP by the plant extract. By contrast, pretreatment with L-NAME significantly (p<0.01) abolished the hypotensive effect of the extract in the rats.

parameter	control	dosage		
		10 mg/kg	20 mg/kg	30 mg/kg
SBP (mmHg)	109 ± 5	76 ± 6 (30.28)	62 ± 10* (43.12)	40 ± 10* (63.30)
DBP (mmHg)	82 ± 7	39 ± 8 (51.85)	31 ± 7* (63.53)	15 ± 7* (81.70)
MAP (mmHg)	91 ±10	51 ± 8 (43.33)	41 ± 6* (55.91)	23.3 ± 5* (67.67)
HR (beats / min)	240 ± 20	100 ± 10 (58.33)	50 ± 10* (71.20)	20 ± 7* (91.60)

The result is expressed as mean ± SEM in 6 observations. The number in parenthesis indicates percentage reduction compared with control. * = P<0.05 when compared to control.

Table 1: Dose-dependent reductions of blood pressure by *Peperomia pellucida* in normotensive rat

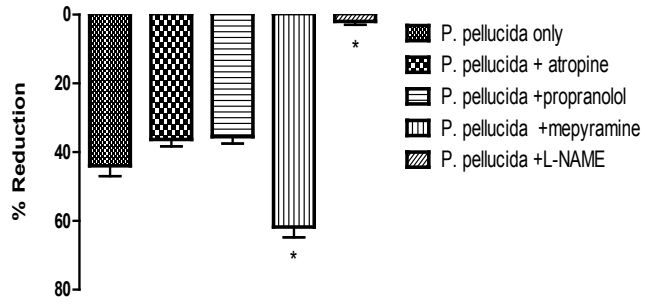

Figure 1: The maximal immediate changes after extract injection in mean arterial pressure (MAP) in anaesthetized rats that received intravenous injection of *Peperomia pellucida* aqueous extract (10 mg/kg). Some animals received an additional pre-treatment of atropine (2 mg/kg), propranolol, or mepyramine (5 mg/kg), L-NAME (5 mg/kg) 5min prior to plant extract administration. Each point represents the mean ± S.E.M. of five rats *P < 0.05 vs. the value without antagonisms.

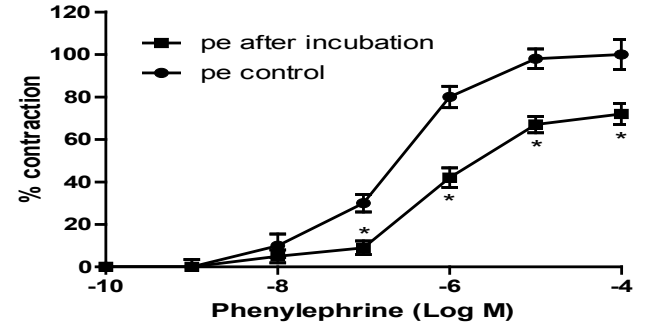

Figure 2: Effect of *Peperomia pellucida* on the concentration-response curves for phenylephrine-induced vasoconstriction of aortic strips. Each data point represents the mean ± SEM *P< 0.05 vs. control.

Effect of *Peperomia pellucida* on phenylephrine-induced contraction

The aqueous extract of *Peperomia pellucida* did not have any vasoconstrictor effect when the aortic rings were incubated init. However, the extract caused a significant (p<0.05) reduction in phenylephrine-induced contraction of aortic rings with a maximum contraction of 72 ± 5% and a rightward shift of the dose-response curve (Figure 2). The sensitivity (pD_2) to phenylephrine in the presence of *Peperomia pellucida* (5.63) was significantly (p<0.05) reduced when compared with the control (pD2 = 6.64).

Effect of *Peperomia pellucida* on relaxation of aorta

The extract of *Peperomia pellucida* (1.9-8.6 mg/ml) caused a dose-dependent relaxation of aortic rings precontracted with phenylephrine (Figure 3). The maximum relaxation to phenylephrine-induced contraction was 33.1 ± 4% in aortic rings with intact endothelium. In endothelium-denuded aortic rings, the vasodilator effect of the extract was completely abolished.

Effect of *Peperomia pellucida* aqueous extract on calcium induced contraction

In the presence of *Peperomia pellucida*, the calcium ion concentration-response curve constructed in a calcium ion free medium on rat aorta was enhanced (Figure 4). There was a significant increase in the maximum contractions to 120.5 ± 4% in the calcium

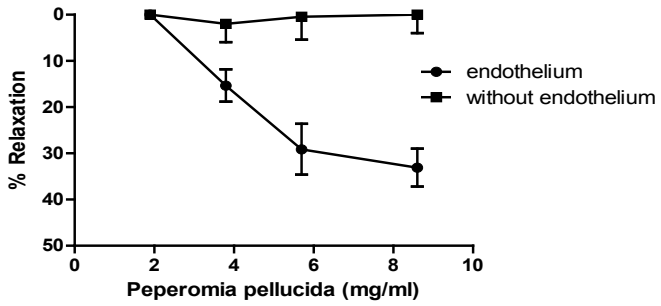

Figure 3: Dose response relaxation curve showing the effect of *Peperomia pellucida* on relaxation of rat aorta with and without endothelium. Mean ± SEM, n=5.

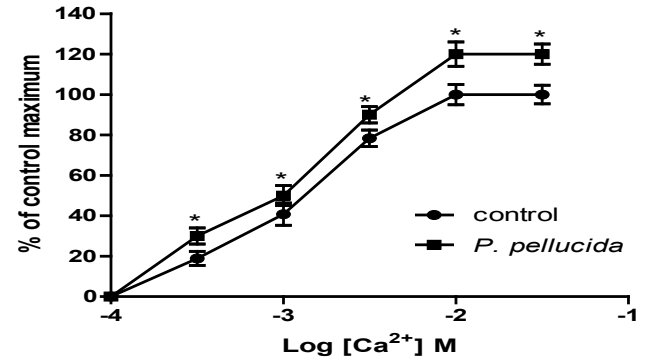

Figure 4: Dose-response curves showing the effect of increasing doses of Ca^{2+} on aqueous extract of *Peperomia pellucida* on dose response curves constructed in Ca^{2+} free medium in isolated rat aorta (values shown are mean± SEM, n =5).

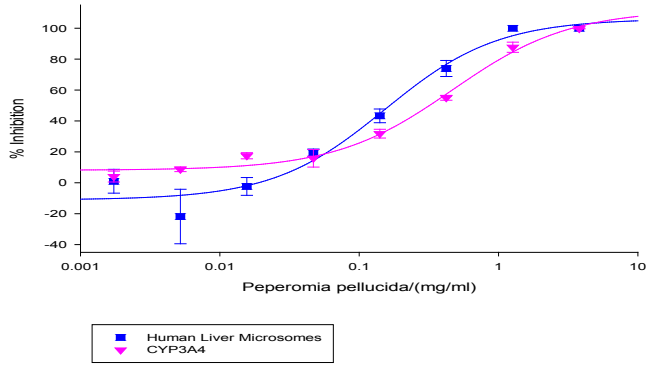

Figure 5: Inhibition of CYP3A4 activity by *Peperomia pellucida* using human liver microsomes and heterologously expressed human enzyme. Human CYP3A4 catalyzed 7-benzyloxy-4-trifluoromethyl-coumarin (50μM) activity was determined in the presence of varying concentrations of the aqueous *P. pellucida* extract (between 0 and 10mg/ml) as described in Materials and Methods. Control enzyme activity (mean ± SEM) for CYP3A4 was 0.09± 0.01 μM/min/pmol of CYP respectively. Data are expressed as mean percentage of control enzyme activity for three independent experiments.

ion induced contractions of the aortic rings incubated with *P. pellucida*, which was significantly ($p<0.05$) higher than that of the control.

Effect of *Peperomia pellucida* extract on CYP enzyme activities

The inhibitory impact of *Peperomia pellucida* extract on the activity of CYP3A4 enzyme is displayed in Figure 5, using both HLMs and heterologously expressed CYP3A4 microsomes and used to generate IC_{50} values which were calculated to be 0.153 ± 0.054 mg/ml and 0.466 ± 0.126 mg/ml, respectively.

Discussion

The major findings of this study are that the aqueous extract of *Peperomia pellucida* produced a dose-dependent decrease in systolic blood pressure, diastolic blood pressure, MAP and heart rate in normotensive rats. The observed fall in BP is in keeping with the traditional use of *Peperomia pellucida* as an antihypertensive agent. The data suggest that the extract had a negative chronotropic effect. Our results also reveal that the muscarinic receptor antagonist, atropine, (atropine may act by blocking the effect of acetylcholine on the heart. Alternatively, atropine may block the action of endothelial acetylcholine acting over the receptor M_3, that induces vasodilation) and the beta blocker, propranolol, (Propranolol, on the other hand, may oppose the vascular smooth muscle relaxation induced by the activation of the beta 2 receptor by endogenous epinephrine) significantly ($p<0.05$) reduced the MAP, while the histaminergic receptor antagonist, mepyramine, did not inhibit the hypotensive effect of the *Peperomia pellucida* extract maybe due to the receptors involved (H_1-receptors mediating contraction and H_2-type receptors relaxation. However, the nitric oxide synthase inhibitor, L-NAME, completely blocked the hypotensive effect and caused a reduction in heart rate. The complete blockade with L-NAME indicated that the plant extract exerts its hypotensive effect via the endothelium-mediated / nitric oxide pathway. This result was further confirmed by the vasodilator action of the extract on endothelial intact rings, which was not seen in endothelium denuded rings.

PPAE caused a significant reduction in phenylephrine-induced contraction of aortic rings and a rightward shift of the dose-response curve; this may indicate a non competitive interaction between PPAE and phenylephrine,

The decreased phenylephrine and Potassium-induced contraction in the aortic rings suggested the involvement of Ca^{2+}. However, in the investigation of the role of Ca^{2+}channels Ca^{2+}- induced contraction in the presence of *Peperomia pellucida* -showed an enhanced calcium-induced contractions. Nitric oxide and cGMP has been reported to inhibit calcium channels as a mechanism of vasorelaxation in vessels [25], our results suggests that the vasorelaxation of blood vessel may not be attributed to the effect of the *Peperomia pellucida* on calcium-mediated antagonism of voltage-stimulated Ca^{2+}channels in the vascular tissues a paradoxical finding. Villar et al. [26] had reported that biflavonoids induced endothelium-dependent relaxation that was unaltered by removal of extracellular calcium, *Peperomia pellucida* is reported to contain flavonoids and essential oils [15] and this may possibly have contributed to the observed effect in this study. The vasodilator effect of *Peperomia pellucida* was observed only in endothelium-intact but not denuded aortic rings, it therefore suggests that the vasorelaxation is endothelium dependent [21,27-29].

Given that *Peperomia pellucida* is used as a complementary medicine and taken along with other medications, it was also important to study its inhibitory potencies against CYP3A4, an enzyme key to evaluating drug interactions. CYPs are responsible for the metabolism of numerous hypertensive therapeutics, including calcium channel blockers, such as diltiazem, felodipine, verapamil amlodipine, lercanidipine, nifedipine, nisoldipine, nitrendipine, HMG-CoA reductase inhibitors such as simvastatin, atorvastatin, cerivastatin, lovastatin and other important classes of drugs including antiretrovirals, immunosuppresants and

antibiotics. Potent inhibition of this enzyme could result in clinically relevant drug adversities. *Peperomia pellucida* aqueous extract inhibited CYP3A4 enzyme activity in both human liver microsomes and heterologously expressed microsomes in the sub milligram/ml levels, which appear to be poor in potency, especially compared with the known potent inhibitor ketoconazole which has an IC_{50} value of $3.1x10^{-5}$ mg/ml. Other medicinal plant extracts have been noted to have IC_{50} values ranging between 0.1 mg/ml [30] to more potent <0.01 mg/ml values [31,32]. Although conclusive determinations can only be drawn from clinical studies, the weak inhibition displayed by the *Peperomia pellucida* extract in this *in-vitro* investigation, is indicative of a fairly low likelihood of clinically observable interactions and adversities through CYP3A4 mediated metabolism.

Conclusion

The results from this study show that the aqueous extract of *Peperomia pellucida* induces hypotension and bradycardia in normotensive rats via nitric oxide dependent mechanisms. *Peperomia pellucida* aqueous extract displayed poor *in vitro* inhibition on CYP3A4 enzyme making it unlikely to impart clinically significant pharmacokinetic drug interactions via the inhibition of this enzyme. This data validate the use of this plant extract as a traditional medicine against hypertension.

Acknowledgement

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Conflict of Interest

The authors declare that there is no conflict of interest.

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