Original Research

Journal of Experimental and Integrative Medicine 2012; 2(3):255-261

In vitro activity-guided vasodilatory effect of *Orthosiphon stamineus* leaves

Omar S.S. Abraika[1], Item J. Atangwho[1,2], Amirin Sadikun[1], Mohd Z. Asmawi[1], Elssanousi A. Hussain[1]

[1]School of Pharmaceutical Sciences, Universiti Sains Malaysia, Penang, Malaysia.
[2]Department of Biochemistry, College of Medical Sciences, University of Calabar, Calabar, Nigeria.

Abstract

Objective: To investigate the vasodilatory effect of *Orthosiphon stamineus* leaves using the activity-guided approach.

Methods: Leaves of *O.stamineus* were serially extracted to obtain the petroleum ether (PE), chloroform (CE), methanol (ME) and water (WE) extracts. The extracts were screened for *in vitro* inhibition of noradrenaline (NA)-induced contraction of rat aorta. The active CE was fractionated and the fractions further tested. The active fraction was screened for chemical composition.

Results: The PE and WE were found to inhibit the NA-induced contractions only at low doses of the agonist, while ME showed no effect. However, the CE inhibited the NA-induced contractions, dose-dependently. At 0.25, 0.5 and 1 mg/ml, CE respectively inhibited the NA contractions by 8.5, 29 and 48%. Of the 5 fractions obtained from CE ($Cf_{1\text{-}5}$), $Cf_{1\text{-}3}$ fractions showed effective dose-dependent inhibition of NA-induced aortic contractions. However, Cf_2 was the most effective; at 0.5 and 1 mg/ml, it inhibited the maximum NA-induced contractions by 16.5 and 49.0%, respectively. Qualitative phytochemical screening of Cf_2 indentified flavonoids as the predominant chemical group in the fraction.

Conclusion: *Orthosiphon stamineus* possesses potent vasodilatory effects *in vitro*, exerted via a possible endothelium-independent mechanism and the flavonoids contained therein, may be responsible.

Key words:
Activity-guided extraction; Flavonoids; Noradrenaline-induced contractions; *Orthosiphon stamineus*; Vasodilatation

Correspondence:
I.J. Atangwho
Department of Biochemistry,
College of Medical Sciences,
University of Calabar,
P. M. B. 1115,
Calabar, Nigeria.
aijustyno@yahoo.com

Received: March 16, 2012
Accepted: April 29, 2012
Published online: June 8, 2012

J Exp Integr Med 2012; 2:255-261
DOI:10.5455/jeim.080612.or.033

Introduction

Orthosiphon stamineus Benth, a Lamiaceae, popularly known in Southeast Asia as 'Misai Kucing' (Malaysia), 'Kumis Kucing' (Indonesia), and in Europe as 'Java Tea', is used traditionally as a remedy for capillary and circulatory disorders (*e.g.* arteriosclerosis), kidney stones, diabetes and nephritis [1]. In Southeast Asia, it is reputable in the traditional management of a variety of ailments, including urinary tract disorders, diabetes mellitus, hypertension, tonsillitis, rheumatism and menstrual disorders, fever, gout and jaundice [2, 3]. *O.stamineus* is listed in the French, Indonesian, Dutch, and Swiss pharmacopoeias for conditions related to renal cleansing and function, and related disorders that include nephritis, cystitis, and urethritis. It is also used as a remedy against high blood cholesterol and blood pressure [4].

Several studies have investigated the biological and pharmacological effects of *O.stamineus* including its diuretic and anti-oxidant effects, as well as its beneficial effects on hyperglycemia and altered lipid profile in diabetic rats [5, 6]. The anti-pyretic and anti-inflammatory effects of the methanol extract have been reported in hyperthermia-induced rats and carraginine inflammed rats paw, respectively [7]. Furthermore, the inhibitory action of the plant extracts against *Vibrio parahaemolyticus* and *Streptococcus mutans* [8] and the hepatoprotective action [9-11] have been demonstrated. Extracts from this plant have been shown to enhance the cytotoxic action of tamoxifen against estrogen-dependent human breast cancer cells by 5-folds, *i.e.* anti-neoplastic effect, as well as suppressing the sprouting neovascularization of microvessels from the excised thoracic rat aorta, implying anti-angiogenic effect [12]. Most recently Mohammed *et al* [13], in a genetotixicity study concluded that the use of *O.stamineus* in traditional medicine possesses no genotoxic risk.

There are currently a number of commercial products derived from this herb. The major identified chemicals of *O.stamineus* are

polymethoxylated flavonoids (*e.g.* sinensetin and eupatorin) and caffeic acid derivatives (*e.g.* rosmarinic acid). The other isolated known chemical constituents of *O.stamineus* are cichoric acid, diterpenes, orthosiphols, monoterpenes, triterpenes, saponins, hexoses, organic acids, and 3'-hydroxy-5,6,7,4'-tetramethoxyflavone [11]. Many of the beneficial effects of *O.stamineus* consumption have been attributed to the pharmacological actions of these major constituents [14].

However, there is hardly any report available in the literature, to our knowledge, of work carried out on the cardiocascular effect of *O.stamineus* to provide a strong rationale or otherwise for its traditional usage in management of cardiovascular diseases such as hypertension. The present study therefore investigated the cardiovascular effect of extracts from *O.stamineus*. The activity-guided approach was used with the aim to understand at least in part, the possible mechanism of action as well as identify the chemical group responsible for the effect.

Materials and methods

Plant material

Fresh leaves of the plant *Orthosiphon stamineus* were collected from Kepala Batas, Seberang Perai, Pulau Penang Island, Malaysia. A voucher specimen (10106) was deposited at the herbarium of School of Biological Sciences, and another voucher specimen (027) deposited at Bilik Herba, School of Pharmaceutical Sciences, Universiti Sains Malaysia**.** The leaves were oven-dried (35-37ºC) to a constant weight. The dried leaves were grounded in an electric grinder to coarse powder (950 g), then packed into aluminium foil sheets of paper and kept in air-tight glass jars. About 600 g of the dried powdered material were extracted successively using a soxhlet apparatus with three solvents namely petroleum ether (60-80ºC), chloroform (45-50ºC) and methanol (60-80ºC). The residue from the methanol extraction, was finally extracted with water by maceration (60ºC)(Fig.1). The extracts obtained from each solvent were then concentrated using a rotary evaporator (45-50ºC) to about 10% of the original volume and thereafter freeze-dried to yield 124 g (20.67%), 67 g (11.17%), 132 g (22.0%) and 74.5 g (12.42%) each of petroleum ether extract (PE), chloroform extract (CE), methanol extract (ME) and water extract (WE), respectively. These extracts were stored refrigerated from where aliquots were withdrawn for the experiments.

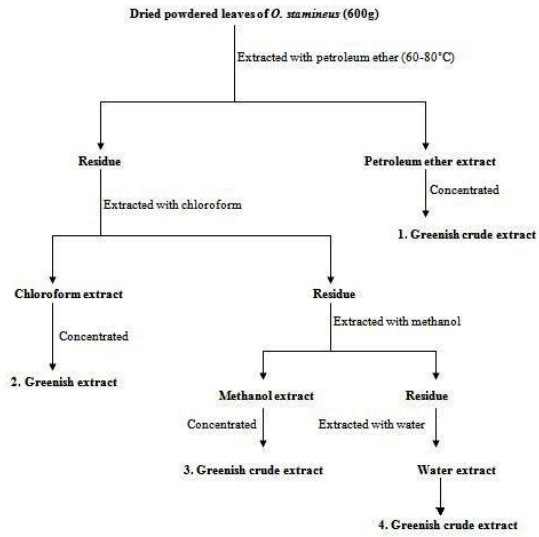

Figure 1: Schematic diagram of successive extraction of dried powdered leaves of *Orthosiphon stamineus*.

Fractionation of the chloroform extract by dry-column flash chromatography

The chloroform extract was found to be the most active in inhibiting noradrenaline-induced contraction of rat aortic strip preparation, therefore it was subjected to further fractionation. Sixty gram of silica gel (Merck, 7730) was packed into a glass column chromatography measuring 27 cm long and 5 cm in diameter fitted with a stopcock. With applied vacuum suction the silica was pressed gently and carefully around the circumference and then towards the center to produce a totally level, and well-compacted bed. The column was then pre-eluted under vacuum with 50 ml (x2) of hexane.

Pre-adsorption and application of the sample

The chloroform extract was pre-adsorbed onto the adsorbent (silica gel) by dissolving 11 g of the extract in 200 ml of chloroform-methanol ($CHCl_3$-MeOH, 1:1) followed by the addition of 20 g of the gel and mixed. Using a rotary evaporator the solvent was evaporated and the resultant dried sample mixture was applied onto the packed column and distributed evenly by applying suction.

Elution

The column was pre-eluted under vacuum with 50 ml (x2) petroleum ether and allowed to dry under suction. Thereafter, it was serially eluted with the following solvents: 450 ml of $CHCl_3$-petroleum ether (30:70); 250 ml of $CHCl_3$-petroleum ether (50:50); 250 ml of $CHCl_3$ (100%); 250 ml of MeOH-$CHCl_3$ (30:70); 250 ml MeOH-$CHCl_3$ (50:50) and finally with 250 ml of MeOH (100%).

DOI:10.5455/jeim.080612.or.033

Fixed-volume fractions were collected in pre-labeled tubes and examined with thin layer chromatography. Fractions with identical RF (Retention Value) were pooled to obtain overall five fractions namely Cf_1, Cf_2, Cf_3, Cf_4 and Cf_5. The fractions were concentrated in a rotary evaporator and further freeze-dried to yield 0.65 g (5.9%), 1.8 g (16.36%), 2.4 g (21.82%), 2.9 g (26.36%) and 2.0 g (18.18%) of Cf_1, Cf_2, Cf_3, Cf_4 and Cf_5, respectively. These were stored in a dissecator until pharmacological screening for their cardiovascular activity.

Animals

Male Sprague-Dawley rats (220-250 g) obtained from the Animal Research and Service Centre (ARSC), Universiti Sains Malaysia (USM), were housed in the animal transit room, School of Pharmaceutical Sciences, USM. They were allowed free access to food (standard laboratory chow, Gold Coin Sdn. Bhd., Malaysia) and tap water. The animals were maintained according to accepted international and national guidelines and the procedure approved by the Animal Ethics Committee of USM (AECUSM).

Experimental procedure

The procedure described previously by Gilani *et al* [15] was used in this study. The rats were sacrificed by cervical dislocation and the aorta quickly isolated and placed in a petri dish containing Krebs solution aerated with 95% O_2 and 5% CO_2 at 37ºC. The preparations were cleaned from fatty tissue and cut into two to three short helical/spiral strips (about 7-10 mm long and 0.8 mm wide). One end of the aortic strip was tied to a tissue holder and then transferred to a 20 ml tissue bath filled with aerated Krebs physiological solution (g/L): NaCl (6.89), $NaHCO_3$ (2.1), $MgSO_4$ (0.29), KCl (0.37), KH_2PO_4 (0.16), glucose (1.1) and $CaCl_2$ (0.28) [16]. The other end of the aortic strip was connected by means of a thread to a force-displacement transducer for isometric recording (Grass Polygraph Model 79D) under a resting tension of 0.5 g. The tissues were pre-contracted with the agonist, noradrenaline (10^{-9} µg/ml), two to three times at the beginning of each experiment in order to reach baseline contraction. Thereafter, successive doses of the agonists (10^{-9}, 3×10^{-9}, 10^{-8}, 3×10^{-8}, 10^{-7}, 3×10^{-7} µg/ml) were added to the bath after the tissues have acquired steady-state equilibrium to the previous dose, until a maximum response/contraction was obtained. This data was used to construct a dose-response curve for the agonist. After the maximum response has been achieved, the agonist was washed off and the tissues allowed to completely relax. Three concentrations of the extracts (0.25, 0.5 and 1 mg/ml) prepared in Krebs solution were then added to the bath and allowed to equilibrate with the tissue for about 20 min. The tissue was again exposed to the agonist and a new dose-response curve obtained by using a range of doses of the agonist, which are sufficient to duplicate the initial dose-response curve. Similar dose-response curves were plotted for a wide range of extract concentrations. The response values obtained from different extract concentrations are expressed as a percentage of the agonist's maximum response (100%). The responses were repeatedly measured for 6-8 times using new tissues and the average of responses to each concentration of the agonist plotted on the ordinate, while the logarithm of the concentration of the agonist on the abscissa. The Krebs solution in the organ bath was replaced with fresh Krebs solution three times every 15-min intervals and the strip allowed to recover for 30 min before the next dose of extract is added.

Phytochemical evaluation of O.stamineus active extract and fraction.

The chloroform extract and Cf_2 fraction, and sinensetin, a standard flavonoid earlier isolated from the leaves of *O.stamineus*, were spotted on thin layer chromatography (TLC) plate and allowed to run using ethyl acetate: chloroform (7:3) as mobile phase. The chromatogram was developed using the natural product poly ethylene glycol (NP/PEG) reagent, and inspected under UV light at 365 nm.

HPLC study of active chloroform extract and standard sinensetin

HPLC analysis of chloroform extract was performed using a Shimadzu-LC system (Japan) equipped with a CBM-20A controller, dual LC-20AT pumps, a DGU-20A5 degasser, an SIL-20A auto-sampler, an SPD-20AV detector and a CTO-10ASvp column oven. Chromatographic separations were achieved using an Agilent Eclipse Plus C18 (250 x 4.6 mm i.d.; 5 µm). A Zorbax guard fittings kit packed with a replaceable Eclipse Plus C18 Guard column (12.5 x 4.6 mm i.d.; 5 µm) was used to protect the analytical column. A reverse-phase HPLC assay was carried out using an isocratic system with a flow rate of 1 ml/min, a column temperature of 25ºC, and a mobile phase of acetonitrile, isopropyl alcohol and 0.02 M phosphate buffer (NaH_2PO_4)(30:15:55, v/v) with pH adjusted to 3.5 using 85% phosphoric acid. The UV detection was set at 340 nm and the injection volume 20 µl. The total run time was less than

20 min for each injection. Data was acquired and processed using LC-Solution Software. The peaks were detected at 340 nm and identified using standard sinensetin.

Analysis of data

One-way analysis of variance (ANOVA) followed by Dunnet′s multiple comparison test was used to compare between groups using the computer software InStat 2. Data were expressed as percentage of the mean ± SEM and comparisons were considered significantly different at $p < 0.05$, $p < 0.01$ and $p < 0.001$.

Results

The effect of crude extracts on noradrenaline-induced contractions of isolated aortic strip preparations

It was found that the NA-induced contractions of the aortic strips were not significantly affected when incubated in the 0.25, 0.5 and 1 mg/ml of methanol extract of *O.stamineus* (Fig.2). The petroleum ether and water extracts (Figs.3&4) showed inhibitory effects on the aortic contractions ($p < 0.05$ and $p < 0.01$, respectively), however, not at high doses of the agonist, NA. On the other hand, the chloroform extract inhibited the NA-induced contractions of the aortic strips at both low and high doses of the agonist ($p < 0.001$)(Fig.5). At maximum response of NA, 0.25, 0.5 and 1 mg/ml of the chloroform extract inhibited the contractions by 8.5, 29 and 48%, respectively. Hence, a potent vasidilatory action.

The effect of fractions of chloroform extract on noradrenaline-induced contraction of isolated aortic strip preparations.

Of the five fractions obtained from the chloroform extract of *O.stamineus*, three (Cf_1, Cf_2 and Cf_3) were found to inhibit significantly adrenaline-induced contractions of the aortic strips, concentration-dependently ($p < 0.05$, 0.01 and 0.001, respectively)(Figs.6-8). Fractions Cf_4 and Cf_5 did not show any significant vasodilatory effects (Figs.9&10). Among the three responsive fractions (Cf_1, Cf_2 and Cf_3), Cf_2 was found to exert the strongest inhibition on noradrenaline-induced contraction of rat aortic strip, hence considered the most effective with vasodilatory action (Fig.7). At 0.5 and 1 mg/ml, Cf_2 inhibited the maximum noradrenaline-induced contraction by 16.5 and 49%, respectively.

Chemical groups present in the O.stamineus active extract and fraction

Inspection of the developed chromatogram under UV light at 365 nm reveals zones of fluorescents emitted by the chloroform extract and the fraction Cf_2 identical to sinensetin, indicating the presence flavonoids as the predominant chemical group (Fig.11). An HPLC analysis of the active chloroform extract of *O.stamineus* compared with pure sinensetin, performed at identical conditions of wavelength (340 nm) and retention time (20 min) also identified sinensetin in the extract (Fig.12). Calculation based on simple linear regression curve revealed that the extract contained 30.907 ± 0.018 µg/mg of sinensetin.

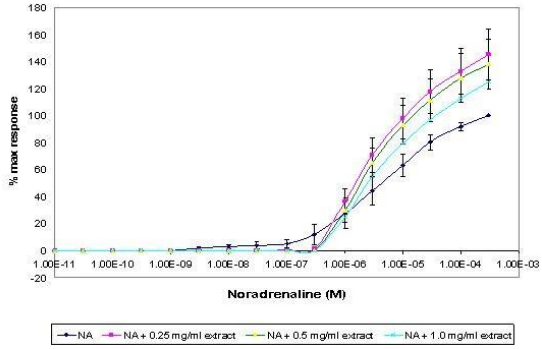

Figure 2. The effect of methanol extract of *O.stamineus* on noradrenaline (NA)-induced contraction of isolated rat aortic strip preparations; n = 6, values are the mean ± SEM)

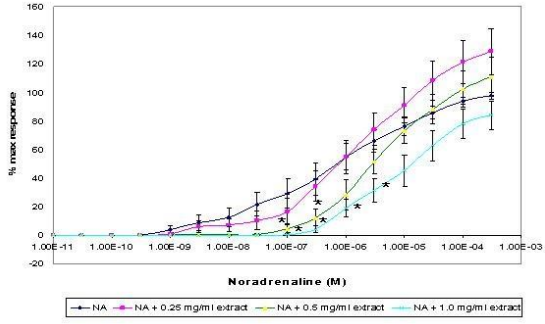

Figure 3. The effect of petroleum ether extract of *O.stamineus* on noradrenaline (NA)-induced contraction of isolated rat aortic strip preparations; n = 6, values are the mean ± SEM, *****$p < 0.05$.

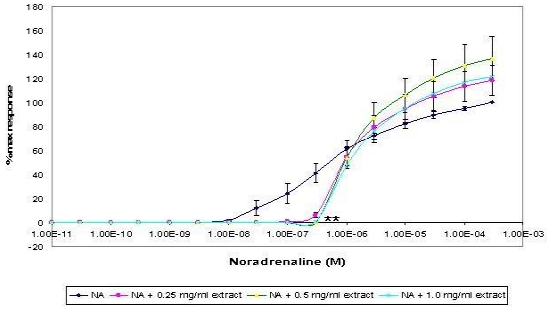

Figure 4. The effect of water extract of *O.stamineus* on noradrenaline (NA)-induced contraction of isolated rat aortic strip preparations; n = 6, values are the mean ± SEM, *****$p < 0.01$.

DOI:10.5455/jeim.080612.or.033

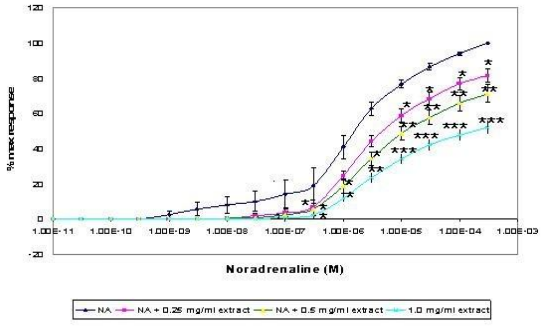

Figure 5. The effect of chloroform extract of *O.stamineus* on noradrenaline (NA)-induced contraction of isolated rat aortic strip preparations; n = 6, values are the mean ± SEM, *****p < 0.05, **p < 0.01 and ***p < 0.001.

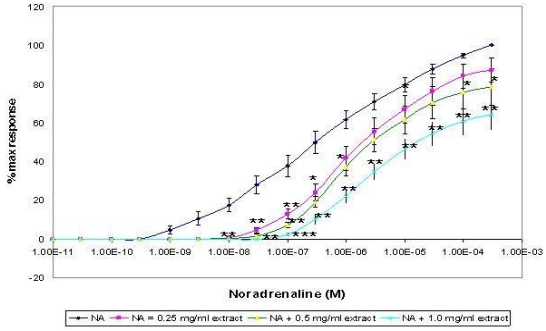

Figure 6. The effect of chloroform fraction Cf_1 of *O.stamineus* on noradrenaline (NA)-induced contraction of isolated rat aortic strip preparations; n = 6, values are the mean ± SEM, *****p < 0.05, **p < 0.01 and ***p < 0.001.

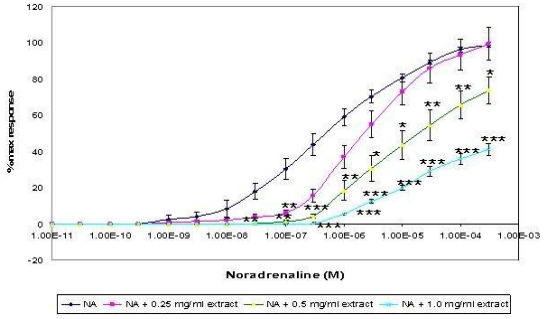

Figure 7. The effect of chloroform fraction Cf_2 of *O.stamineus* on noradrenaline (NA)-induced contraction of isolated rat aortic strip preparations; n = 6, values are the mean ± SEM, *****p < 0.05, **p < 0.01 and ***p < 0.001.

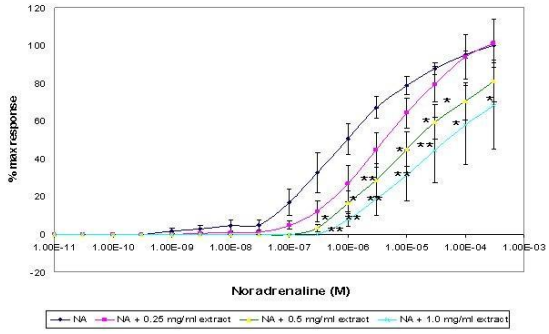

Figure 8: The effect of chloroform fraction Cf_3 of *O.stamineus* on noradrenaline (NA)-induced contraction of isolated rat aortic strip preparations; n = 6, values are the mean ± SEM, *****p < 0.05, **p < 0.01 and ***p < 0.001.

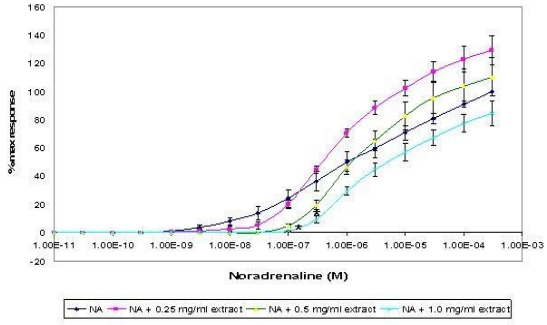

Figure 9. The effect of chloroform fraction Cf_4 of *O.stamineus* on noradrenaline (NA)-induced contraction of isolated rat aortic strip preparations; n = 6, values are the mean ± SEM, *****p < 0.05.

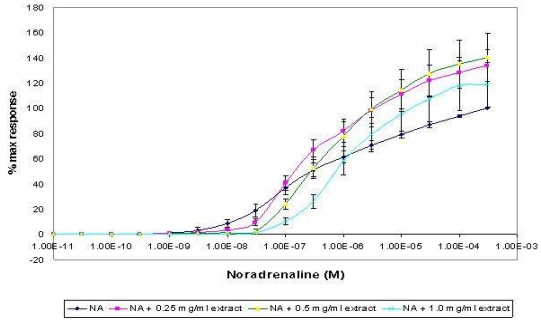

Figure 10: The effect of chloroform fraction Cf_5 of *O. stamineus* on noradrenaline (NA)-induced contraction of isolated rat aortic strip preparations; n = 6, values are the mean ± SEM.

Discussion

Cardiovascular diseases are increasingly becoming one of the leading diseases causing morbidity and mortality in Malaysia and, indeed, the Southeast Asia. In the traditional system, *Orthosiphon stamineus* is widely employed as a remedy against these cardiovascular related diseases including hypertension, diabetes mellitus, arteriosclorosis, *etc* [2-4]. The present investigation was therefore carried out to provide a pharmacological rationale, if any, for this traditional practice. A simple *in vitro* method in assessing the ability of the extract to inhibit/vasodilate noradrenaline-induced contraction of an isolated rat aorta was used [15]. In the first phase of the experiments, it was found that the petroleum ether, chloroform and water extracts of

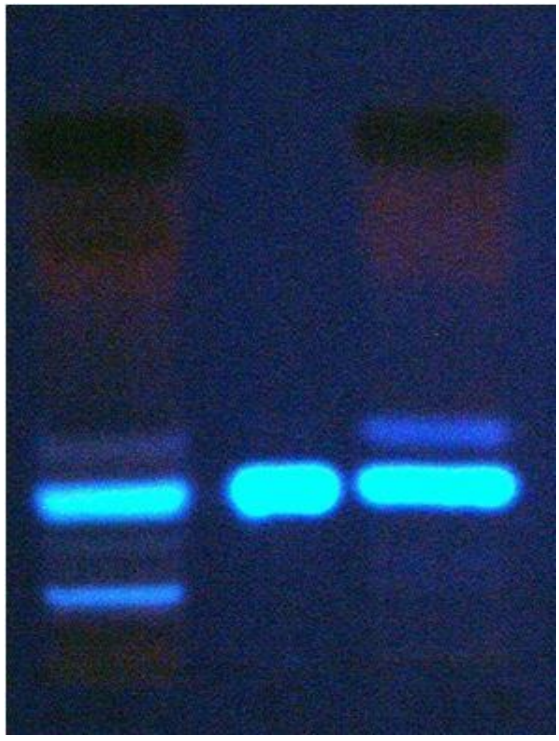

Figure 11. Thin layer chromatography (TLC) profile of chloroform extract $CHCl_3$, fraction Cf_2 of *O.stamineus* and sinensetin flavonoid (Sin) using ethyl acetate: chloroform (7:3) as mobile phase after spray with natural product polyethylene glycol NP/PEG reagent and viewed under UV_{365} light.

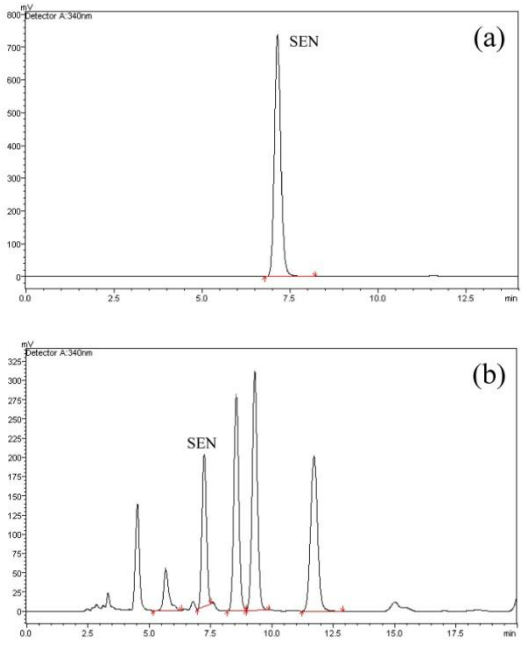

Figure 12. HPLC chromatogram of **(a)** standard sinensetin (SEN) and **(b)** chloroform extract of *O.Stamineus*; peaks SEN are indicated.

Orthosiphon stamineus (but not the methanol extract), inhibited the noradrenaline-induced contraction of the isolated aorta. Of these three extracts, chloroform extract was the most effective in inhibiting the noradrenaline-induced contraction of the aorta, implying a potent vasodilatory activity. This observation suggests that the chemical groups responsible for the observed activity are predominant in the chloroform extract. Hence, the chloroform extract was further fractionated and the fractions obtained tested for vasodilatory effect.

The result obtained for these fractions showed that the fraction Cf_2 was the most potent in vasodilating the noradrenaline-induced contraction of the aorta, which also implied that the active chemical groups were predominant in this fraction. These compounds were found in a qualitative TLC screening using a suitable mobile phase to be, flavonoids, most likely sinensetin. The qualitative assay result was supported with an HPLC analysis of the chloroform extract which revealed the amount of sinensetin in the extract to be about 30.907 ± 0.018 µg/mg. Although various chemical constituents have been isolated from the *O.stamineus* plant, the report on flavonoids in general, and sinensetin in particular, has been very consistent [1, 11]. The flavonoids (sinensetin) may be responsible for the observed vasodilatory effects, considering the fact that they were detected in the most effective fraction Cf_2. In line with our submission, an earlier study by Duarte *et al* [17] indicated the direct vasodilatory effects of flavonoids on vascular smooth muscle *in vitro*. The claimed antihypertensive effect of *O.stamineus* extract can be attributed to the flavonoids contained therein.

It is likely from our observations in this study that *O.stamineus* exerts the vasodilatory action via mechanisms that are independent of the endothelium. Noradrenaline is known to produced contractions via the β_1-adrenergic receptor, which works through the adenylate cyclase-cAMP system to activate G_s-protein and eventually protein kinase A [18]. Also, according to Bolton *et al* [19] the NA-induced contractions are resistant to calcium-free conditions, and calcium-free conditions are mainly caused by mechanisms other than the opening of potential-sensitive calcium channels (entry and release of stored calcium). If NA elicits contraction by mechanisms which do not involve opening of potential-sensitive calcium channels, then by the same token the *O.stamineus* extract attenuation of NA-induced contraction must involve some mechanism other than opening

DOI:10.5455/jeim.080612.or.033

potential-sensitive calcium channels. Hence, implicating an endothelium-independent mechanism. Moreover, Duarte *et al* [17] had in a preliminary investigation found that the *in vitro* vasodilatory effects of flavonoids are mainly endothelium-independent. In a later detailed study, reported that the main mechanism of flavonoid-induced vasodilatation results from the inhibition of protein kinases such as myosin light chain kinase (MLCK) and, possibly, other kinases involved in Ca^{2+}-sensitizing mechanisms, *e.g.* protein kinase C and cyclic nucleotide phosphodiesterases [20]. There is therefore a high chance that *O.stamineus* may exert vasidilatory effect by a shared mechanism of the flavonoids.

In conclusion, although this work can only be considered as exploratory, taken together, evidence from this study suggests that *O.stamineus* contains a compound or a group of compounds with a cardiovascular activity on the aorta. This group of compounds is likely to be the flavonoids. The observed vasodilating properties of these compounds may be exerted via an endothelium-independent mechanism. *O.stamineus* appears to be a potential natural source of ingredients for the management of cardiovascular disorders, hence warrants further research.

Acknowledgements

The authors are sincerely grateful to the Academy of Science for the Developing World (TWAS) and the Universiti Sains Malaysia (USM) for the joint TWAS-USM fellowship offer to Item Justin Atangwho, which contributed immensely to the completion of this research work.

Conflict of interest

The authors declare that they have no conflict of interest.

References

1. Tezuka Y, Stampoulis P, Banskota AH, Awale S, Tran KQ, Saiki I, Kadota S. Constituents of the Vietnamese medicinal plant *Orthosiphon stamineus*. Chem Pharm Bull (Tokyo) 2000; 48:1711-9.
2. Awale S, Tezuka Y, Banskota AH, Adnyana IK, Kadota S. Nitric oxide inhibitory isopimarane-type diterpenes from Orthosiphon stamineus of Indonesia. J Nat Prod 2003a; 66:255-8.
3. Chin JH, Abas HH, Sabariah I. Toxicity study of orthosiphon stamineus benth (misai kucing) on Sprague dawley rats. Trop Biomed 2008; 25:9-16.
4. Ahamed Basheer M, Abdul Majid A. Medicinal potentials of *Orthosiphon Stamineus* Benth. WebmedCentral CANCER 2010; 1:WMC001361.
5. Sriplang K, Adisakwattana S, Rungsipipat A, Yibchok-anun S. Effects of *Orthosiphon stamineus* aqueous extract on plasma glucose concentration and lipid profile in normal and streptozotocin-induced diabetic rats. J Ethnopharm 2007; 109:510-4.
6. Arafat OM, Tham SY, Sadikun A, Zhari I, Houghton PJ, Asmawi MZ. Studies of diuretic and hypouricemic effects of Orthosiphon stamineus methanol extract in rats. J Ethnopharm 2008; 118:354-60.
7. Yam MF, Asmawi MZ, Basir R. An investigation of the anti-inflammatory and analgesic effects of *Orthosiphon stamineus* leaf extract. J Med Food 2008; 11:362-8.
8. Ho CH, Noryati I, Sulaiman SF, Rosma A. In vitro antibacterial and antioxidant activities of *Orthosiphon stamineus* Benth. extracts against food-borne bacteria. Food Chem 2010; 122:1168-72.
9. Yam MF. Antioxidant and hepatoprotective effects of *Orthosiphon stamineus* Benth standardized extract. Am J Chinese Med 2007; 35:115-26.
10. Faizul FM, Aminudin N, Kadir HA, Tayyab S. Bilirubin lowering potential of *Orthosiphon stamineus* in temporarily jaundiced adult rats. Afr J Pharm Pharmacol 2009; 3:359-61.
11. Alshawsh MA, Abdulla MA, Ismail S, Amin ZA. Hepatoprotective effects of *Orthosiphon stamineus* extract on thioacetamide-induced liver cirrhosis in rats. Evid Based Compl Altern Med 2011; 2011:103039.
12. Sahib HB, Aisha AF, Yam MF, Asmawi MZ, Ismail Z, Salhimi SM, Othman NH, Abdul Majid AMS. Anti-angiogenic and antioxidant properties of *Orthosiphon stamineus* Beth. methanolic leaves extract. Int J Pharmacol 2009; 5:162-7.
13. Muhammad H, Gomes-Carneiro MR, Poça KS, De-Oliveira AC, Afzan A, Sulaiman SA, Ismail Z, Paumgartten FJ. Evaluation of the genotoxicity of *Orthosiphon stamineus* aqueous extract. J Ethnopharm 2001; 133:647-53.
14. Pan Y, Abd-Rashid BA, Ismail Z, Ismail R, Mak JW, Pook PC, Er HM, Ong CE. *In vitro* effects of active constituents and extracts of *Orthosiphon stamineus* on the activities of three major human cDNA-expressed cytochrome P450 enzymes. Chem Biol Interact 2011; 190:1-8.
15. Gilani AH, Shaheen F. Vasoconstrictory and cardiotonic effect of *Haloxylon recurvum* extract. Phytother Res 1994; 8:115-7.
16. Kitchen I. Textbook of *In Vitro* Practical Pharmacology, Blackwell Scientific, London, UK, pp101-118, 1984.
17. Duarte J. Jimenez R, Villar IC, Vizcaino F, Jimenes J, Tamargo J. Vasorelaxant effects of the bioflavonoid chrysin in isolated rat aorta. Planta Med 2001; 67: 567-9.
18. Lieberman M, Marks AD. Mark's Basic Medical Biochemistry: A Clinical Approach, Third Edition, Lippincott Williams & Wilkins, New York, NY, USA, pp 495-496, 2009..
19. Bolton TB, Lang RJ, Takewaki T. Mechanisms of action of noradrenaline and carbachol on smooth muscle of guinea-pig anterior mesenteric artery. J Physiol 1984; 351:549-72.
20. Duarte J, Perez-Vizcaino F, Jimenez J, Tamargo J, Zarzuelo A. Flavonoids and cardiovascular diseases. In: Rahman A (ed) Studies in Natural Products Chemistry, Volume 25, Part F, Elsevier, Amsterdam, Netherlands, pp 565-605, 2002.