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Antihypertensive and Vasorelaxant Effect of *Alstonia scholaris* Stem Bark Extracts and Fractions

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ABSTRACT
Alstonia scholaris (AS) have been widely studied for its several pharmacological benefits and has been used in folklore medicine to lower blood pressure. The present study was carried out to verify the antihypertensive and vasorelaxant effect of *A. scholaris*. Male Spontaneously Hypertensive Rats (SHRs) were divided into four groups; Two control groups (positive and negative control) and two test groups of six rats (n = 6). Test groups were given daily oral administration of the methanolic (ASME) and water (ASWE) extracts (1000 mg kg^{-1} day^{-1}) of *A. scholaris* while positive and negative control groups received Verapamil (15 mg kg^{-1}) and water respectively for 2 weeks. Vasorelaxant effect of AS extracts and its fractions were also evaluated on Endothelium-intact and denuded rat aortic ring preparations pre-contracted with either Phenylephrine (PE, 1 μM) or KCl (80 mM). Daily oral administration of the methanol extract (1000 mg kg^{-1} for 2 weeks) exhibited a significant decrease in blood pressure ($p<0.05$) in SHR rats compared to aqueous extract. All extracts and fractions (0.125-4 mg mL^{-1}) gave a dose-dependent vasorelaxation on endothelium-intact aortic rings pre-contracted with phenylephrine (1 μM) or KCl (80 mM). *Alstonia scholaris* (AS) showed blood pressure lowering activity in *in vivo* rat model and vasorelaxation effect on pre-contracted aortic rings possibly via endothelium independent mechanisms that need further evaluation. The result also indicates its potent negative chromotropic and ionotropic effect which also augment its antihypertensive property.

Key words: Antihypertensive, vasorelaxation, SHR, hypertension, *Alstonia scholaris*

INTRODUCTION

Cardiovascular diseases (CVD) have been the most endemic global disease that causes death of millions per year. According to World Health Organization (WHO) report, an estimate of 17 million deaths is recorded annually resulting from CVD with hypertension being the leading CVD accounting for 55% of all cases (WHO., 2013).

Hypertension damages major organs, eventually leading to heart failure, vascular injury, micro-vascular functional impairment, atherosclerosis, renal dysfunction, ischaemic and haemorrhagic stroke, myocardial infarction, chronic kidney disease, cognitive decline and premature death (Chobanian *et al*., 2003; Cuddy, 2005). Untreated or sub-optimally controlled hypertension is usually associated with a progressive rise in blood pressure which leads to increased cardiovascular, cerebro-vascular and renal morbidity and mortality (Hobbs, 2004; Leoncini *et al*., 2003).

Eighty percent of the cases of CVD occur in developing countries (Ibrahim and Damasceno, 2012), where there is a high reservation among the populace on the use of current conventional medications, preferring traditional and

herbal medications for treatment (Farnsworth, 1980; Neergheen-Bhujun, 2013; WHO., 1998). This preference for herbal medicine has result in recent surge in interest in the pharmacological evaluation of ethnomedicinal plants for their therapeutic proficiency in the treatment of various ailments (Janes, 1999; Pal and Shukla, 2003).

Alstonia scholaris (AS), a genus of the family Apocynaceae, is a native of the Indian subcontinent and Southeast Asia. It is considered as an all-around herbal plant and its folklore uses in the prevention and treatment of various diseases has been well documented (Baliga, 2012; Pratap *et al*., 2013; Dey, 2011). The plant has been investigated for its antidiabetic and antihyperlipidemic effects (Arulmozhi *et al*., 2010); anti-inflammatory and analgesic effects (Shang *et al*., 2010); antimalarial (Gandhi and Vinayak, 1990) and antimicrobial (Bonvicini *et al*., 2014; Mahapatra and Banerjee, 2010) among other diseases. The concoction of the dried bark is used extensively to treat respiratory and cardiovascular diseases such as asthma, hypertension, lung cancer and pneumonia, whereas an infusion of the leaves is used as a remedy for fever (Kumar *et al*., 2014; Baliga, 2010; Stocklin, 1986). Recent studies have also reported the potent broncho-dilatory effect of the ethanol extract of *A. scholaris* in the anaesthetized rats (Channa *et al*., 2005). Despite the ancient uses of this plant for various pulmonary and cardiovascular complications in traditional medicine, no systemic study has been conducted to evaluate its antihypertensive potentials. The aim of this study is to provide a pharmacological validation for the traditional use of AS in the treatment of hypertension using both *in-vivo* and *in-vitro* experimental models.

MATERIALS AND METHODS

Preparation of leaf extract: The bark of *Alstonia scholaris* (AS) and few leaves for identification was obtained from the penang forest, Malaysia, 3 km away from penang hill (coordinates 5.4246° N, 100.2689°E) in September, 22, 2013. The plant's leaf was submitted to the Herbarium at the school of biological sciences, Universiti Sains Malaysia (USM), for authentication (specimen voucher registration No.: 11479). About 2.5 kg of air dried and powdered stem bark of *Alstonia scholaris* (AS) was defatted with petroleum ether (60-80°C) to remove the fat, latex and high molecular non-polar compounds. The defatted plant residue was extracted by maceration in methanol for 24 h with intermittent stirring at 45°C. The dried macerate after methanol extraction was drenched in water for few hours to get water extract (ASWE). Each solvent was changed several times until no more coloration is observed. The extracts obtained with each solvent were filtered through Whatman filter paper (No. 1). The filtrates were concentrated in a rotor evaporator under vacuum and the concentrated extract was dried in a freeze dryer and later in oven (45°C). The methanol extract (ASME) was subjected to liquid-liquid fractionation successively to obtain Dichloromethane (DCF), Ethyl acetate (EAF), n-butanol (NBF) and water (AQF) fractions.

Drugs and chemicals: The methanol, n-butanol, ethylacetate and dichloromethane solvents where purchased from HB SDN (Butterworth, penang, Malaysia). Verapamil chloride, acetylcholine and phenylephrine were purchased from sigma-aldrich Company (St Louis, Mo, USA). All drugs and chemicals used were of analytical grade.

Animals: The Spontaneous Hypertensive Rats (SHRs) were obtained from the USM Animal Research and Service Center (ARSC) responsible for inbreeding experimental animals for research purposes in the school of pharmacy. The SHRs, 16-20 weeks old (weighing 250-300 g) were kept in the animal transit room of the School of Pharmaceutical Sciences, USM. Animals were acclimatized to laboratory conditions for 7 days. During acclimatization, prior to commencing the experiments, the SHRs were trained to stay in the restrainer every day for tail-cuff blood pressure measurement. The animals were maintained on a 12-h light/12-h dark cycle during this study with access to food (standard rat diet Gold Coin) and water *ad libitum*. The handling and use of animals was in accordance with the institutional animal ethic guidelines (Animal Ethics Committee, School of Pharmaceutical Sciences, Universiti Sains Malaysia).

Experimental protocol

***In vivo* experiment:** SHR were randomly divided into four groups of six rats each (n = 4×6) and were treated as follows: Group 1 received Verapamil drug (15 mg kg^{-1}) and serve as the reference group. Group 2 received the vehicle (distilled water) and served as negative control. Group 3 and 4 were treated with methanol and water extracts of AS respectively by oral gavages at a dose of 1000 mg kg^{-1} body weight per day for a fortnight.

Blood Pressure (BP) of the rats was measured by tail cuff method using a CODA non-invasive blood pressure monitoring system. The BP was measured before starting the experiments (D0), one hour after oral administration on day one (D1), day seven (D7) and day fourteen (D14).

Vasorelaxation effect of the plant extracts (Isolated rat aorta ring experiment): In the *in vitro* experiment, the rats were euthanized with Carbon dioxide (CO_2). A midline incision was made through the sternum to open up the thoracic cavity and excise the aorta. Excised aorta was immediately placed in a Kreb's-Ringer-Bicarbonate (KRB) solution (118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 4.2 mM $NaHCO_3$, 2 mM $CaCl_2$, 10 mM glucose, pH 7.4), cleansed of fat and connective tissues gently and sectioned into rings of approximately 3.0 mm long. The rings were suspended on a tissue bath containing 10 mL of standard

KRB solution continuously aerated with carbonated oxygen (carbogen, 95 O_2 and 5% CO_2) and maintained at 37°C. The aortic rings were maintained under a 1.0 g tension and allowed to equilibrate for 45-60 min. KRB solution was replaced after every 15 min during equilibration. Contraction of aorta rings was induced with phenylephrine (PE, 1 μM) or potassium chloride (KCl, 80 mM). The tension was measured using force-displacement transducer (model FT-03) coupled with a data acquisition system (AD Instrument, Sydney, Australia).

The presence of functional endothelial was assessed by the ability of ACh (10 μM) to induce more than 60% relaxation of aortic rings precontracted with PE (1 μM). In some experiments, the endothelium was removed mechanically by gentle abrasion of the intimal surface of the aortic rings with forceps. Endothelium denuded rings were assessed when Ach (10 μM) elicited less than 10% relaxation in aortic rings precontracted with PE (1 μM).

Each extract/fraction was assayed in 6 aortic rings at each tested concentration. The tension attained following contraction induced with PE or KCl (80 mM) and concentration response relaxation following cumulative addition of AS extract/fractions was recorded. Relaxation, a measure of inhibition of contraction in aortic ring pre-contracted with phenylephrine was measured in percentage and calculated as follows:

$$\text{Relaxation}(\%)=\left(\frac{\text{Tc}-\text{Tt}}{\text{Tc}}\right)\times 100$$

where, Tc stands for change tension after contraction with phenylephrine or KCl, while Tt stands for change in tension after adding extract. Values were expressed as Mean±Standard Error of Mean (SEM).

Statistical analysis: Statistical evaluation was done using two-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test (DMCT) by using Graphpad Prism statistical software (version 6.0). The significance level was set at $p<0.05$, $p<0.01$ and $p<0.001$.

RESULTS

Effect of oral administration of AS on SHR blood pressure and heart rate (*In vivo*): Daily oral administration of AS extracts shows a significant decrease in the blood pressure by both the water (ASWE) and methanol (ASME) extracts. ASWE did not cause any noteworthy decrease in both the systolic and diastolic blood pressure on D1 (Fig. 1a). However, a significant decrease in the systolic blood pressure was observe with a net decrease of -17.5±0.65 mmHg and -18.6±1.98 mmHg on D7 and D14, respectively when compared to the baseline value (D0) ($p<0.01$).

The methanol extract of Alstonia scholaris (ASME) was more effective as it elicited a mild decrease in both the systolic (SBP) and diastolic (DBP) blood pressure on D1 ($p<0.05$) with its effect being more apparent on the SBP. Change in the blood pressure was significantly high on D7 with a net reduction of -17.4±1.5 and -19.8±2.8 mmHg ($p<0.001$) for the DBP and SBP, respectively. The effect of ASME peaked on D14 with -20.3±1.8 mmHg for DBP and -24.7±2 mmHg for SBP.

The reference group treated with verapamil (15 mg kg^{-1}) also shows a significant decrease in SBP and DBP at -15.7±5.2 mmHg and -16.71±6 mmHg ($p<0.01$) on D1 (Fig. 1). Peak effect of verapamil on the SBP (-33.92±9.3 mmHg) and DBP (-30.2±9.8 mmHg) was observed on D14 ($p<0.001$). Changes in the measured hemodynamic parameters of the negative control group were rather insignificant.

After 14 days of consecutive oral administration, Water extract of Alstonia scholaris (ASWE) shows no significant decrease in the HR on D1 compare to the initial value ($p<0.05$). However, a slight, statistically insignificant effect on the HR was observed on D7 and D14 ($p<0.05$). Interestingly, ASME did not give a significant decrease in the HR on D1 ($p<0.01$), however, a significant decrease was observed on D7 which peaked on D14 ($p<0.001$). This is in congruence with the changes we observed in our reference group treated with the calcium channel blocker, verapamil which gives a precipitous reduction in the HR of treated rats on D7 and D14 ($p<0.001$).

The ASME treated group shows no statistically significant difference in all the hemodynamic parameters measured when compared to the verapamil treated group (positive control) ($^{\#}p<0.05$) on the respective days. In contrast, ASWE treated group showed a significant difference in the DBP on D7 ($^{\#}p<0.05$) and D14 ($^{\#\#}p<0.01$); in SBP on D14 ($^{\#}p<0.05$); in MAP on D7 ($^{\#}p<0.05$) and D14 ($^{\#\#}p<0.01$) and in Heart rate (HR) on D7 and D14 ($^{\#}p<0.05$) when compared to similar parameters in verapamil treated group. Furthermore, no statistically significant difference was observed between the ASWE and ASME treated groups (Fig. 2).

Effect of AS extracts on PE-induced and KCl-induced tonic contractions in endothelium-intact and -denuded rat aortic rings: In the *in vitro* experiments to assess the vasodilatory effect of AS extracts on aortic ring preparations, AS extracts (0.125-4.0 mg) were cumulatively added after contraction of suspended aortic rings with PE (1 μM) or KCl (80 mM). Cumulative addition of ASME extract (0.125-4.0 mg) elicited a concentration-dependent relaxation of aortic rings pre-contracted with PE with a maximal effect (R_{max}) of 96.3±4.9% (EC_{50} = 1.8 mg mL^{-1}) at 4 mg mL^{-1} (Table 1). In contrast, ASWE did not show any significant effect on the vascular tone of the pre-contracted aortic with an R_{max} of 13±0.67% (Fig. 1).

Fractions of ASME were also assayed *in vitro* for their vasorelaxation effect. The n-butanol fraction of the *A. scholaris* methanolic extract (i.e., NBF-ASME) elicited the

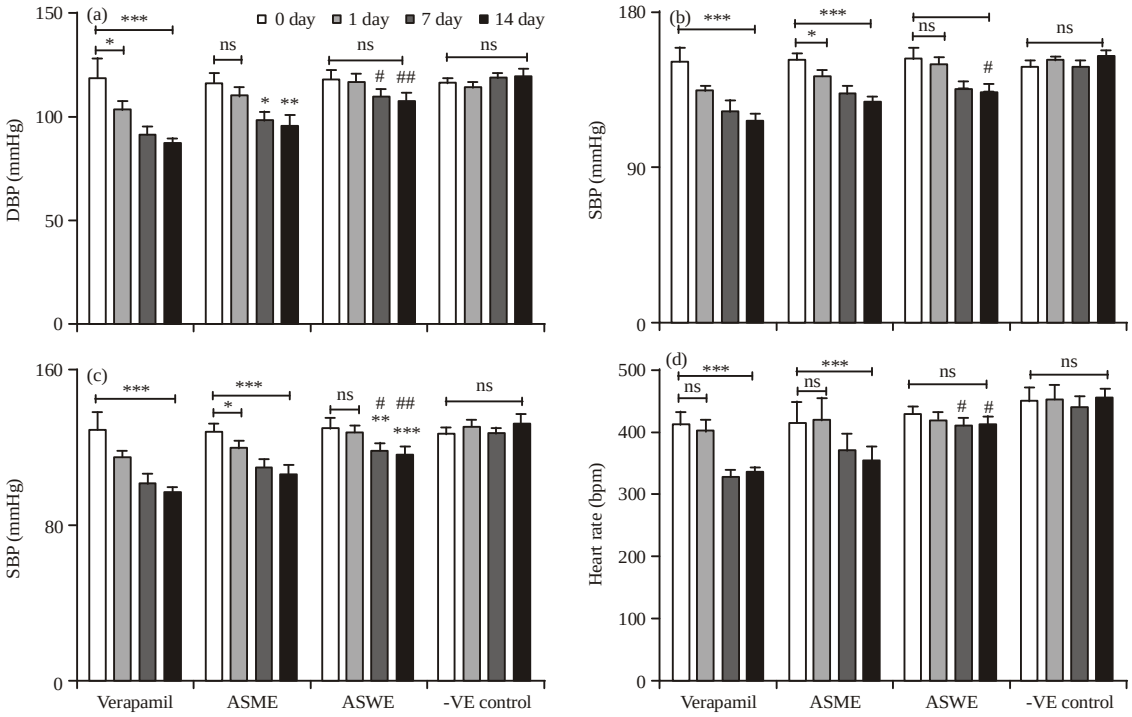

Fig. 1(a-d): Effect of oral administration of the methanol extract (ASME), aqueous extract (ASWE) of Alstonia scholaris stem bark (1000 mg kg^{-1}) and Verapamil (15 mg kg^{-1}) on (a) Diastolic (DBP), (b) Systolic (SBP), (c) Mean arterial blood pressure and (d) Heart rate of SHR rats. Data is expressed as Means±SEM for six (n = 6) determinations. *p<0.05, **p <0.01, ***p<0.001 when compared to baseline values (D0 of treatment), #p<0.05, ##p<0.01 when compared with verapamil group of treatment

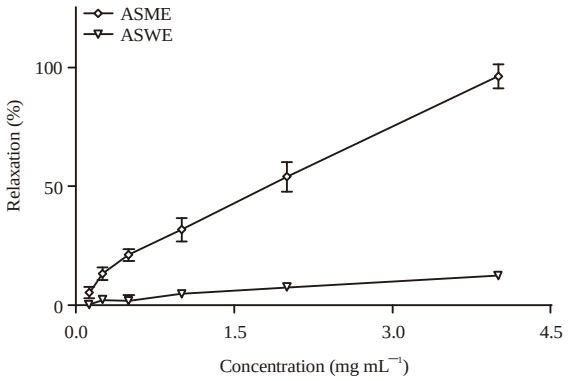

Fig. 2: Vasorelaxant response induced by *Alstonia scholaris* extracts on rat aortic rings pre-contracted with phenylephrine (1 μM). Data is expressed as Means±SEM for six (n = 6) determinations

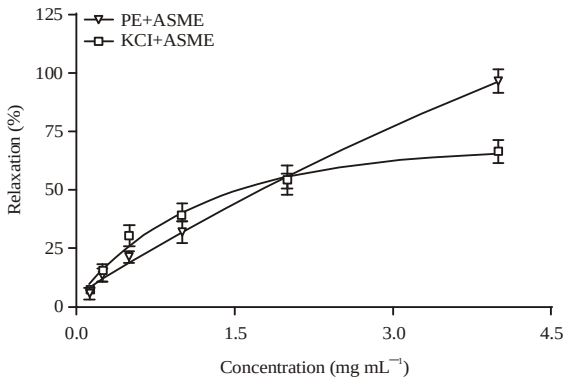

Fig. 3: Vasorelaxant response induced by *Alstonia scholaris* methanol extracts on rat aortic rings pre-contracted with phenylephrine (1 μM) or KCl (80 mM). Data is expressed as Means±SEM for six (n = 6) determinations

highest concentration-dependent inhibitory effect on aorta ring pre-contracted with PE (1 μM) with R_{max} of 106.4±0.045% and an EC_{50} of 0.74 mg mL^{-1} (Fig. 3). This was followed by the ethyl acetate fraction (i.e., EAF-ASME) with R_{max} 89.2±0.08% and EC_{50} of 1.33 mg mL^{-1}. The R_{max} values for DCF-ASME and AQF-ASME were 57±0.062 and 56±0.08% with both fractions eliciting EC_{50} >1.5 mg mL^{-1} at the maximum tested concentration for each fraction. On the whole, the disparity in the R_{max} values was significant (p = 0.005) (Fig. 4). The concentration dependant relaxation effect of different arations of ASME shown in Fig. 5.

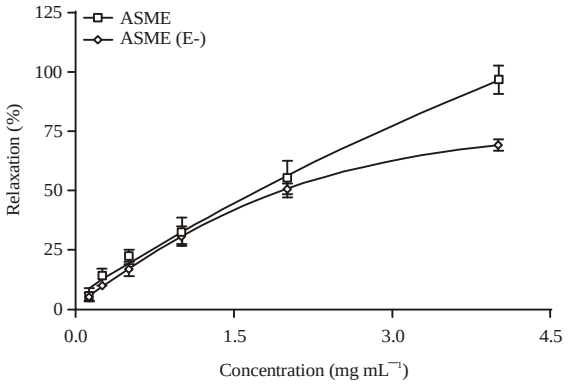

Fig. 4: Vasodilator effect was evaluated on endothelium-intact and endothelium-denuded preparations. Data is expressed as Means±SEM of 6 experiments.*p = 0.05 and **p = 0.001 denote a significant difference between the R_{max} values (unpaired t test)

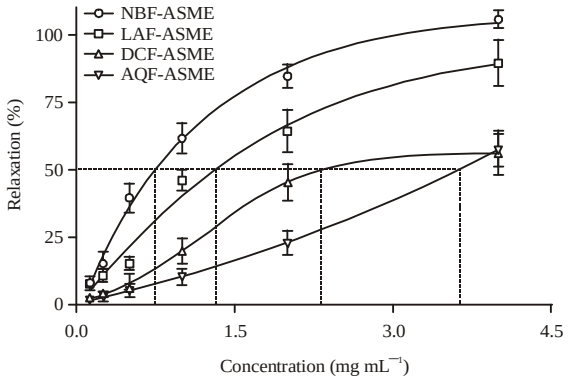

Fig. 5: Concentration-dependent relaxation effect of different fractions of ASME methanol extract on vascular activity of endothelium-intact aortic rings pre-contracted with PE (1 μM). Data is expressed as the Mean±SEM of six (n = 6) aorta ring experiments

DISCUSSION

The rationale of treating hypertension is to reduce the risk of cardiovascular complications, such as stroke and heart attack (Turnbull, 2003). The results obtained from this study have demonstrated that the extracts from *A. scholaris* stem bark (ASE) possess blood pressure lowering effect on Spontaneous Hypertensive Rats (SHR). The methanol extract showed more potency than the water extract in lowering the blood pressure. Reduction in blood pressure was also observed in the reference group treated with verapamil, a calcium channel antagonist. The Heart Rate (HR) represents the number of times the heart beats per minute (Robertson, 1987). Abnormally high HR known as tachycardia has been identified as a cardiovascular risk factor (Cook *et al*., 2006; Escobar and Barrios, 2008), that is associated with diseases like hypertension (Fitzpatrick *et al*., 2001; Soato and Krieger, 1974) atherosclerosis (Eller, 2007), coronary heart disease (Anonymous, 2011) and it is a risk factor for hypoxia (Zuzewicz *et al*., 1999). HR monitoring is also used as a good prognostic indicator in the clinical diagnoses of patients predispose to heart attack (Sandset *et al*., 2014). ASME gave a progressive gradual decrease in the HR of the treated rats. This correlates with the effect of verapamil on the HR of the reference group which significantly decreased. Verapamil is known to exert a negative chronotropic, inotropic and dromotropic effect on the heart (Grossman and Messerli, 1997, 2004). Thus, these results suggest that ASME may possess negative inotropic and chronotropic effects on the heart. These kinds of phenomena have generally been shown in hearts treated with antihypertensive agents, such as calcium channel blockers (Little and Cheng, 1994) and angiotensin converting enzyme inhibitors (Raddino *et al*., 1991).

Table 1: EC_{50} and R_{max} values for extracts and fraction of *Alstonia scholaris* on endothelium-intact and denuded aortic rings precontracted with PE. Values expressed as Mean±SEM of six (n = 6) aorta ring experiments

Extracts	R_{max} (%)	EC_{50} (mg mL^{-1})
ASWE	13.0±0.670	-
ASME	96.3±4.900	>1.80
DCF-ASME	57.0±0.062	>1.50
EAF-ASME	89.2±0.080	1.33
NBF-ASME	106.4±0.045	0.74
AQF-ASME	56.0±0.080	>1.50

ASWE: Water extract, ASME: Methanol extract, DCF-ASME: Dichloromethane fraction, EAF-ASME: Ethyl acetate fraction, NBF-ASME: N-butanol fraction and AQF-ASME: Water fraction

In evaluating the vasorelaxant effect, ASE elicited concentration-dependent relaxant effects on both PE and KCl-precontracted endothelium-intact and endothelium-denuded aortic rings with ASME being more potent. However, the relaxation effects on the aorta smooth muscle were less pronounced against KCl-induced contraction and endothelium-denuded aortic rings. Also, the relaxant effects of ASME on endothelium-denuded aortic rings were reduced. These findings suggested that the relaxant effect caused by ASE was both endothelium-dependent and endothelium-independent.

ASME fraction also showed relaxant effect of PE-induced rat aorta smooth muscle contraction with the n-butanol fraction as the most potent fraction followed by water (NBF-ASME), ethylacetate (EAF-ASME), Dichloromethane (DCF-ASME) and Water (AQF-ASME) fractions. Worth noting is the increase in the relaxant effect of the fractions when compared to the parent methanol extract, an indication that the partitioning process has concentrated the active constituents, particularly, in the n-butanol and ethyl acetate fractions.

The constituents responsible for the potent antihypertensive and vasorelaxant effects of *A. scholaris* have not yet been identified. However, in the preliminary phytochemical analysis we carried out on ASME and its fractions, we detect the present of Reducing sugar, alkaloids,

Table 2: Phytochemical analysis of the methanol extract of *Alstonia scholaris* and its fractions

Parameters	ASME	EAF-ASME	NBF-ASME	AQF-ASME
R. sugar	+++	-	++	+++
Alkaloids	+++	+	++	+
Tannin	++	-	+	+
Flavonoids	++	++	-	-
Glycosides	++	++	+	-
Terpenoids	+	-	+	-
Saponin	++	+		++

tannins and Glycosides in moderate and high abundance in the n-butanol and ethyl acetate fractions (Table 2). Flavonoids were also detected in the ethyl acetate fraction but absent in n-butanol fraction. A number of alkaloids and flavonoids have been reported to exert pharmacological effect on the blood pressure (Cassidy *et al*., 2011; McGregor and Segel, 1955; Balasuriya and Rupasinghe, 2011). Alkaloids isolated from Roufinia serpentine reduced the blood pressure in patients with hypertension (Herbeuval *et al*., 1954; Loffler *et al*., 1953). Bioflavonoids such as quercetin and quercitrin have been detected in *A. scholaris* (Hui *et al*., 2009) and have shown cardiovascular relaxant effects with the flavonoid quercetin one being more studied (Chen and Pace-Asciak, 1996; Roghani *et al*., 2004; Zhou *et al*., 2006).

The presence of glycosides may also play a vital role in the effect of ASME on the heart rate observed. Cardiac glycosides have been used for decades for the treatment of congestive heart failure (Schoner and Scheiner-Bobis, 2007) and have been reported to reduces sinoatrial firing rate (decreases heart rate; negative chronotropy) and reduces conduction velocity of electrical impulses through the atrioventricular node (negative dromotropy) (Eick and Hoffman, 1969). This may be associated with the reduction in the heart rate observed in ASME treated rats. These findings strongly suggest the possible involvement of phytoconstituents from this class in the observed antihypertensive and vasorelaxant activity of our active extracts and fractions. Thus, it seems that the direct vasorelaxant and possible negative ionotropic and chronotropic effects of ASME may contribute to alleviate the development of hypertension. On the other hand, while ASWE shows no effect on the heart rate or direct vasodilation effect, a significant decrease in BP, albeit less potent than ASME, was observe in the *in vivo* study. It can be deduced that ASWE has an indirect effect on the hemodynamic parameters to cause blood pressure lowering effect.

CONCLUSION

The result we observed from this study has validated and provided a pharmacological base evidence for the folklore use of Alstonia scholaris in the treatment of hypertension among many other uses. Our research group is currently studying the pharmacological mechanisms underlying the antihypertensive and Vasorelaxant effect of the active extracts with the prospect of eventual isolation, identification and physicochemical characterization of the bioactive constituent.

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