JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY ADVANCES

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J Phys Pharm Adv 2014, 4(6): 379-388

Online version is available on: www.grjournals.com

Original Article

Pharmacodynamic Evaluation of *Hibiscus Sabdariffa* Extract for Mechanisms Underlying its Antihypertensive Action: Pharmacological and Biochemical Aspects

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Abstract

The aim of the present study was to investigate the mechanisms by which *Hibiscus sabdarifa* petal extract produces its well-established antihypertensive activity, from both pharmacological and biochemical aspects of view. Pharmacologically, the aqueous extract exhibited a hypotensive effect when injected intravenously (femoral vein) in anesthetized rats cannulised *via* femoral artery and connected to pressure transducer. This hypotensive effect was resistant to prior administration of different pharmacological blockers, including atropine (0.2 mg/250 g. b. wt.), hexamethonium (2 mg/250 g. b. wt.), propranolol (0.1 mg/250 g. b. wt.) and phentolamine (0.1 mg/250 g. b. wt.). The aqueous extract showed concentration-dependent relaxation of noradrenaline-precontracted aortic rings using tension recording technique. This relaxation was resistant to tetrodotoxin (neuronal blocker; 0.25 μM), L-NAME (Nitric oxide synthase inhibitor; 200 μM), indomethacin (cyclo-oxygenase inhibitor; 10 μM) nifedipine (Ca^{++} channel blocker; 10 μM), apamin (small conductance K^+ channel blocker; 1 μM), glibenclamide (ATP-sensitive K^+ channel blocker; 100 μM) and endothelial denudation. Biochemically, the aqueous extract of Hibiscus petals exhibited significant decrease in serum levels of ACE, total cholesterol, tri-acylglycerols, LDL and VLDL as well as Na^+ concentration in hyperlipidemic rats; but raised the level of HDL; with no significant effect on serum K^+. The obtained results may indicate that Hibiscus petal extract produces its antihypertensive action *via* four mechanisms; first is direct relaxing the vascular smooth muscle; the second is decreasing the level of ACE in serum; the third is decreasing the elevated blood lipid profile; while and the fourth is decreasing serum Na^+ level.

Keywords: ACE, aorta, antihypertensive, hibiscus, lipid profile.

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Received on: 07 Mar 2014
Revised on: 02 May 2014
Accepted on: 22 May 2014
Online Published on: 13 Jun 2014

Introduction

Phytotherapy occupies an important place in health care throughout the world. Herbal drugs are more preferred than allopathic drugs because of their efficacy, easy availability and causing less or no side effects. Hypertension is a health problem affecting large population all over the world and dangerously is a progressive and fatal disease (Williams, 1995). Therefore, it is a respected target for both pharmacological and non-pharmacological methods of control. Among the former, is the herbal medicine that currently became of a considerable importance, especially in chronic and life lasting disease conditions like hypertension.

Hibiscus sabdariffa L. (HS) is a herb belonging to the family *Malvaceae* (Bako *et al.*, 2010) that is growing globally and is cultivated for its leaf, fleshy calyx, seed or fibre (Dalziel, 1973). In addition to Roselle, in English speaking regions, it is also called as Karkadé or Carcadé in North Africa and the Middle East. HS has been, for many years, used in many countries as an antihypertensive agent, however, its exact mechanism of action remained as controversial matter.

Previous studies concluded that HS caused bradycardia and hypotension in normotensive rats and exhibited negative inotropic and negative chronotropic effects on isolated guinea pig atria (Tiamjan, 1999). Obiefuna *et al.,* (1993) found that petal extract of HS produced relaxation of isolated rat aorta and that relaxation was partly due to inhibition of Ca^{++} influx through receptor gated channels.

However, Adegunloye *et al.,* (1996) showed that the inhibition by HS does not appear to be mediated by sympathetic inhibition but may be mediated via nitric oxide (NO) release. A third point of view was suggested by Mojiminiyi *et al.*, (2012) that HS attenuation of salt-induced hypertension may be associated with its high K^+ content or high potassium: sodium ratio and not with altered pressor/depressor response to noradrenalin or acetylcholine. Additional data reported by Agbai *et al.,* (2013) suggested that HS extract increased plasma ADH and reduced plasma Na^+, Cl^-, $HCO3^-$ and K^+. For established hypertension, diuretics, adrenoceptor blockers, calcium channel blockers and angiotensin converting enzyme inhibitors are useful (Zare, 1991; Williams, 1995; Rezvani, 1999). However, the antihypertensive actions of these drugs are usually associated with side effects such as vertigo, depression, hallucinations, tachycardia, angina, hypokalemia, gastrointestinal disturbances and leukopenia (Williams, 1995). Therefore, eyes have been targeted to the use of natural herbal medicines to control hypertension like olive leaf, garlic (Harenberg, 1978) and HS in particular (Haji Faraji *et al.*, 1999; Onyenekwe *et al.*, 1999; Wang *et al.*, 2000; Odigie *et al.*, 2003).

Hypercholesterolemia, resulting from cholesterol metabolic alterations, is a major cause of cardiovascular disease associated with hypertension (Keys, 1970; Kannel *et al.*, 1971). Previous studies reported that HS extract decreased elevated serum cholesterol levels in rabbits (Chen *et al.*, 2003) and rats (Chen *et al.*, 2004) fed on diets with high cholesterol content.

In the present study, we tried to bring the pharmacological and biochemical concepts together regarding the mechanism(s) underlying the antihypertensive action of HS extract both *in vivo* and *in vitro* studies.

Materials and methods

Animals

Forty male Wistar rats aged 12 weeks old (about 250 grams of body weight) were used for conducting different experiments of the present study. Before experimentation, rats were acclimatized to light-dark cycles of 12 hours in an air-conditioned room at a temperature of 25 °C. Rats during acclimatization had free access to water and given either basal (for pharmacological experiments) or fat-enriched (for biochemical experiments) diet for induction of hyperlipidaemia that is associated with hypertension. Fat in fat-enriched diet was given as 1% cholesterol and 2% coconut oil.

Twenty rats were assigned to the biochemical experiment. Twelve rats fed on basal diet were assigned for in vivo pharmacology experiment. The

remainder of rats were also fed on basal diets and assigned for in vitro pharmacology experiment.

HS Extract

Enough amount of dry HS petals was purchased from our local market. An aqueous extract was prepared by two methods; the first one is by maceration of the dried petals of HS in distilled water over night at room temperature in a sealed suitable container. While the second method was done by digestion of HS petals in distilled water by heat (56 °C for 60 minutes) then cooling to room temperature.

Aqueous extracts prepared by either maceration or digestion gave the same results, and thus referred as aqueous extract. Extraction processes were done by immersing 20 g of HS dried petals in 200 ml of distilled water. Clear HS extracts were obtained by straining using a mesh and then filtration of the strained solution using Whatman filter paper No. 1 (11 μm). The filtered solutions were used in both in vivo and in vitro experiments. The yield percent after straining and evaporating the extract was 49.25%.

Drugs and Chemicals

Norepinephrine, acetylcholine, L-NAME, indomethacin, apamin, tetrodotoxin, atropine, hexamthonium, phentolamine, propranolol, nifedipine and glibenclamide have been purchased from Sigma, Saint Louis, USA. Cholesterol has been purchased from Al-Gumhurria Chemical Co., Cairo, Egypt.

In Vivo Pharmacological Study

The method modified after (Jackson, 1939) was adopted. Rats were anaesthetized using thiopental intraperitoneally (equivalent to intravenous route) in a dose of 40 mg/kg of body weight half an hour before the experiment. An anaesthetized animal was placed on its back under a dissecting microscope while its head and limbs were fixed down to the rubber floor of the dissecting plate.

A piece of skin about 1 x 2 cm. was cut free from the inguinal region close up by the abdominal wall. The subcutaneous fat was carefully torn away with two forceps exposing a vein leading to the skin. This was tied out and cut. The femoral vein was then exposed and the portion between the junction with the saphenous vein and the inguinal ligament was very carefully cleared free of connective tissue. A fine plastic cannula (1.5 mm. external diameter drawn out to 0.25 to 0.5 mm.) was connected via a rubber tube with a syringe containing saline solution which allows injection of HS extract followed by saline to wash it all in. The whole unit was filled with heparinized saline and all air bubbles were excluded. The femoral vein was tied at its junction with saphenous vein to leave about 1 to 1.5 cm. of the distended vein ready for cannulation. A slight tension was placed on the vein and cannulated.

The cannula was pushed in a further cm. and firmly tied in place. The femoral artery of the other side can be then cannulated. To prevent clots, 0.1 mL of heparin (5000 units/mL) was injected intravenously through the cannula already inserted and washed in with 0.2 mL of saline. A plastic cannula, slightly larger than the venous one was connected to a blood pressure transducer (MLT0699, AD instruments, Australia) via a tube containing heparin solution to prevent blood coagulation and connected with a thermal-array recorder (RTA-1100M, Nihon Kohden, Tokyo, Japan) for illustration and further analysis.

The whole unit was filled with heparinized saline and care must be taken to exclude any air bubbles. The femoral artery was separated from the nerves and vein and carefully freed of connective tissue. A fine thread (No. 5/0) was placed around it to be firmly tied after insertion of the cannula. After insertion and fixation of the cannula in situ, the unit has been tested to ensure that there were no leaks. The trace of systemic blood pressure was recorded immediately before and at different time intervals following intravenous injections of 0.2 mL of HS extract with or without certain antagonists.

The antagonists used were 0.2 mg atropine (as 0.1 mL of 2 mg atropine sulphate/mL), 0.1 mg propranolol (as 0.1 mL of 1 mg propranolol hydrochloride/mL), 0.1 mg phentolamine (as 1 mg phentolamine mesylate/mL) and 2 mg hexamethonium (as 0.1 mL of 20 mg hexamethonium bromide/mL). Enough wash out periods were left in between the different tests and this was indicated by retaining the normal blood pressure trace.

In Vitro Pharmacological Study

Aortic tissue preparation: Rats were killed under light ether anaesthesia and thoracic aorta was dissected out. The vessel was transferred immediately into Krebs' solution (see next) that is aerated and its temperature was kept at 37 °C. Under a dissecting microscope, aorta was gently flushed with the solution and then cut into rings of 5 mm length with care to avoid damage of the endothelium. In some rings, endothelium was intentionally damaged using fine forceps. A ring was mounted in 10 ml-capacity organ bath filled with Krebs' solution; where 2 fine stainless steel wires were passed through it.

The lower wire was fixed into the bottom of the bath, while the upper one was attached to a force displacement transducer and a computer unit for recording (MLT0420, AD instruments, Australia). Using a manipulator, the smooth muscle of the ring was subjected to 1 g tension. The ring was left to equilibrate for 60 min, norepinephrine (NE; 2 μM) was applied to contract the smooth muscle of the mounted ring. Upon the plateaued contraction, aliquots of 0.1 mL of HS extract were applied on to the aortic ring preparations dissected from the used rats to investigate its relaxant effect and to locate its site of action. In some experiments, endothelium-devoid rings were used. Integrity or damage of the endothelium was checked by application of acetylcholine (ACh; 10 μM) upon the plateaued NE-induced contraction; relaxation of the ring by Ach indicates integrity of the endothelium and vice versa. All changes in muscular tension were recorded.

Physiological solution: The physiological solution used in this study had the following composition in mM: NaCl 118, KCl 4.6, CaCl2 2.7, MgCl2 1.2, KH2PO4 1.2, NaHCO3 25, glucose 11. The solution in the supply reservoir was gassed continuously with 95% O2: 5% CO2 gas mixture creating a pH of 7.2 and was warmed to 37 °C.

Biochemical Study

Twenty male rats were used for this experiment. They were randomly divided into three groups (6, 7 and 7 rats). The first month was for conditioning by keeping rats on either basal (control group) or high-fat (hyperlipidemic group and hyperlipidemic-treated group) diets. On the second month, the first group (6 rats) that was kept on basal diet received 1 mL normal saline via gastric tube once daily. The second group (7 rats) that was kept on high-fat diet was given 1 ml normal saline via gastric tube once daily. While the third group (7 rats) that was fed on high-fat diet was given 1 ml HS aqueous extract via gastric tube once daily for a month. Blood samples were taken every week along the second month of the experimental course, 2 hours post last administration of the corresponding week. Blood samples were collected from the retro-orbital venous plexus at the medial canthus of the eye by means of heparinized capillary tubes. The collected blood was allowed to clot at room temperature, and then the formed clot was allowed to shrink by refrigeration for one hour. Clear sera were separated by centrifugation at 3000 r.p.m. for 10 minutes and then collected by an automatic pipette. Serum samples were kept in Eppendorf's tubes in -20 °C for further analysis. Serum ACE, Na+, K+, total cholesterol, tri-acylglycerols and lipoproteins were measured using kits supplied by commercial kits according to procedure supplied by the manufacturer.

Statistical Analysis

Data were expressed as a mean ± S.E. The value of "n" indicates the number of animals (in vivo) or experiments (in vitro) performed. Each in vitro experiment was repeated at least three times. Data were compared by ANOVA using SPSS software; and difference was considered significant at $P < 0.05$.

Results

As shown in figure 1, intravenous injection of 0.2 ml of HS aqueous extract exhibited lowering of both systolic and diastolic blood pressures (about 25 mm/Hg as observed from the tracing). This hypotensive effect was resistant to prior conditioning of the anesethized rat with different pharmacological blockers including the cholinergic blocker atropine (0.2 mg/250 g rat), β-adrenergic blocker propranolol (0.1 mg/250 g rat), α-adrenergic blocker phentolamine (0.1 mg/250 g rat) and the ganglionic blocker hexamethonium (2

mg/250 g rat) (figure 2). Two µM-precontracted aortic ring preparations showed (figure 3) dramatic relaxation upon application of 100 µL of HS aqueous extract.

The relaxant effect of HS extract markedly increased upon its cumulative application. Repeating the same experiment using preparations pre-incubated with different putative blockers did not impede the relaxant effect of HS extract. The used blockers were the neuronal blocker TTX (250 nM), the nitric oxide synthase blocker L-NAME (200 µM), the calcium channel blocker nifedipine (10 µM), the small conductance potassium channel blocker apamin (1 µM), the ATP-sensitive potassium channel blocker glibenclamide (100 µM) and the cyclo-oxygenase blocker indomethasin (10 µM). The relaxation was also evident in preparations with denuded epithelia.

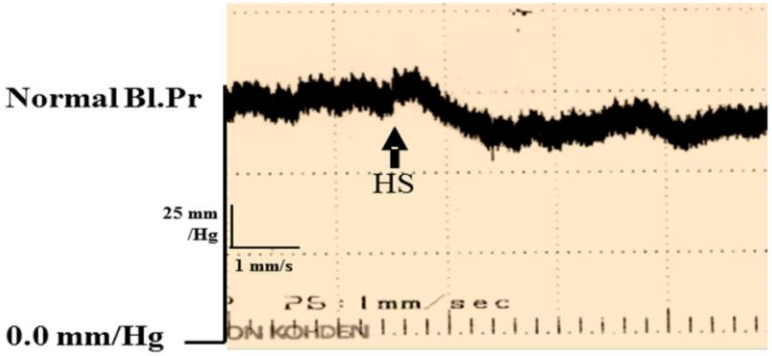

Figure 1:
Typical tracing representing the normal systolic and diastolic blood pressures recorded by cannulation of femoral artery of rat and the effect of iv injection of 0.25 mL HS aqueous extract via femoral vein.

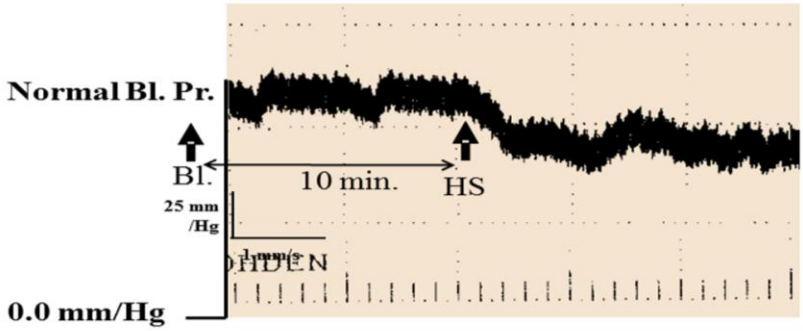

Figure 2:
Typical tracing representing the effect of prior injection of different pharmacological blockers as atropine, propranolol, phentolamine, and hexamethonium (Bl.) on the hypotensive effect of iv injection of 0.25 mL HS aqueous extract; this tracing is that of atropine 0.2 mg/Kg of body weight.

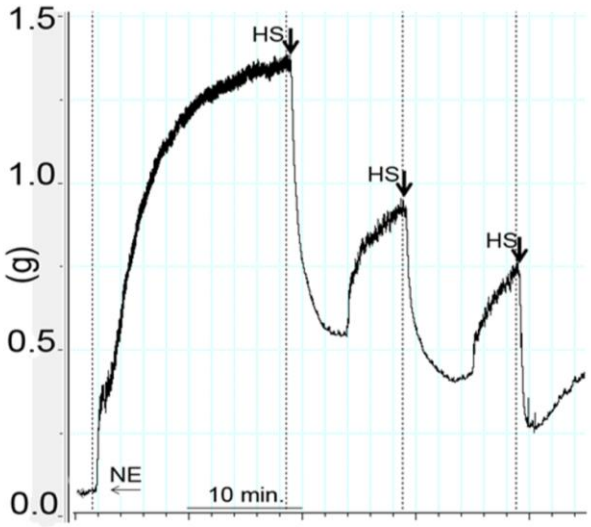

Figure 3:
Typical tracing representing the effect of 0.1 mL HS aqueous extract on the precontracted aortic rings (2 µM of norepinephrine).

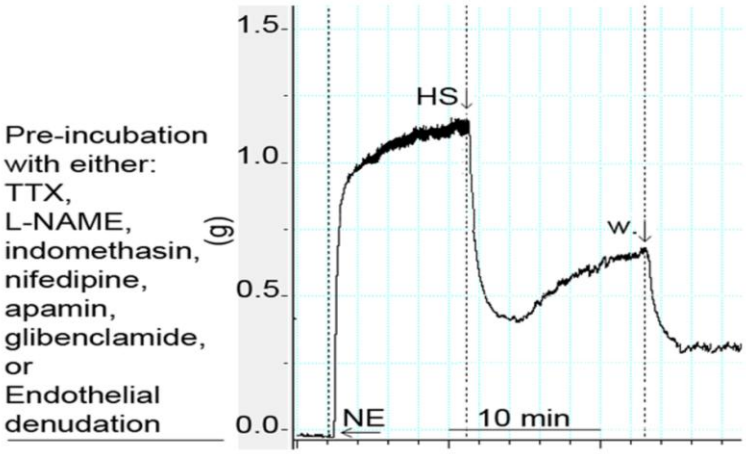

Figure 4:
Typical tracing representing the effect of 0.1 mL HS aqueous extract on the precontracted aortic rings (2 µM of norepinephrine) in presence of one of the different pharmacological antagonists (TTX, L-NAME, indomethasin, nifedipine, apamin, glibenclamide) or subjected to endothelial denudation; this tracing is after L-NAME as an example.

HS aqueous extract significantly decreased the elevated lipid parameters in hyperlipidemic rats namely, cholesterol, tri-acylglycerols, low- and very low-density lipoproteins. In contrast, it improved the decreased level of high density-lipoproteins.

These effects were time dependent during a month experimental course (table 1). In the same animals, HS extract decreased the elevated levels of ACE (table 2) as well as Na+ with no significant effects on K+ serum levels.

Table 1: Effect of oral administration of *Hibiscus sabdarifa* aqueous extract on selected lipid parameters in albino rats rendered hyperlipidemic by keeping on high-fat diet (Mean ± SE; n = 6 in the control group & 7 in the other two groups).

Week	Parameter Group	TAG (mg/dL)	TC (mg/dL)	HDL (mg/dL)	LDL (mg/dL)	VLDL (mg/dL)
First week	Control	79.99±7.69	70.94±9.52	41.66±3.14	19.28±8.93	15.99±1.53
	Hyperlipidemic	111.10±4.44[a]	157.13±8.24[a]	33.80±3.14[a]	89.10±4.38[a]	22.22±0.88[a]
	HS extract-treated	101.10±11.75	152.38±12.59	38.47±1.19[b]	81.68±12.13	20.22±2.35
Second week	Control	81.55±4.44	71.42±8.24	40.47±4.29	21.83±5.16	15.11±0.88
	Hyperlipidemic	124.44±4.44[a]	166.66±9.52[a]	31.61±4.29[a]	94.15±6.44[a]	24.88±0.88[a]
	HS extract-treated	111.11±16.02[b]	157.14±8.24	36.04±1.19[b]	80.87±9.66[b]	21.22±3.20
Third week	Control	83.66± 7.69	73.66±12.59	39.28±2.06	24.04±9.08	17.33±1.53
	Hyperlipidemic	133.33±15.39[a]	171.42±8.24[a]	29.23±2.38[a]	99.51±3.32[a]	26.66±3.07[a]
	HS extract-treated	115.55±19.37[b]	152.38±4.75[b]	36.90±4.76[b]	82.36±12.84[b]	22.11±3.87
Fourth week	Control	85.10± 11.75	76.71±8.25	40.04±3.14	27.44±8.95	18.22±2.35
	Hyperlipidemic	159.99± 7.69[a]	190.47±4.76[a]	25.99±2.06[a]	108.47±2.34[a]	31.99±1.53[a]
	HS extract-treated	124.44± 8.89[b]	147.62±12.59[b]	36.66±2.38[b]	81.07±9.01[b]	24.89±1.77[b]

[a] differs significantly from control; [b] differs significantly from hyperlipidemic ($P \leq 0.05$).

Table 2: Effect of oral administration of *Hibiscus sabdarifa* aqueous extract on salt-related ions and ACE in albino rats rendered hyperlipidemic by keeping on high-fat diet (Mean ± SE; n = 6 in the control group & 7 in the other two groups).

Week	Parameter Group	Sodium (nmol/mL)	Potassium (nmol/mL)	ACE (U/L)
First week	Control	120.96 ±2.79	2.31 ± 0.26	7.14 ± 0.14
	Hyperlipidemic	161.28 ±10.57[a]	2.96 ± 0.19	11.96 ± 0.29[a]
	HS extract-treated	156.45 ±5.81	2.89 ± 0.19	9.71 ± 0.26[b]
Second week	Control	121.39 ±5.05	2.38 ±0.21	7.33 ± 0.52
	Hyperlipidemic	162,90 ±14.33[a]	2.53 ± 0.33	12.76 ± 0.24[a]
	HS extract-treated	155.02 ±4.75	2.67± 0.24	9.11 ± 0.14[b]
Third week	Control	125.79 ±2.79	2.38 ± 0.21	7.84 ± 0.22
	Hyperlipidemic	173.51 ±13.38[a]	2.96 ± 0.14	13.66 ± 0.17[a]
	HS extract-treated	153.22 ±8.98[b]	2.46 ±0.40	9.55 ± 0.31[b]
Fourth week	Control	112.90 ±7.02	2.24 ±0.19	8.16 ± 0.55
	Hyperlipidemic	174.52 ±5.88[a]	2.67 ±0.26	14.97 ± 0.23[a]
	HS extract-treated	153.22 ±5.81[b]	2.17 ±0.12	9.85 ± 0.24[b]

[a] differs significantly from control; [b] differs significantly from hyperlipidemic ($P \leq 0.05$).

Discussion

The need to control hypertension and associated conditions is very imperative as its complications may be associated with mortality. The use of conventional drug control of hypertension is associated with many side effects besides, it is not cost-effective. Heavy responsibility is placed on pharmacologists and pharmacists to search for better cost-effective and safer antihypertensives (Oates *et al.*, 2001). For the moment, effective control for hypertension is achieved by a combination therapy, using both chemical and herbal drugs. The second party was taken as a complementary medicine to decrease the dose of the classical chemical drug. This may be a midway step on the road of the use of herbal remedy that can be used as an absolutely alternative medicine. This may be an appreciated target as hypertension is an only controllable, non-treatable life companion. Therefore, this control is needed to be natural, safe, cost-effective and always available.

Data of the present study provided a strong evidence for the use of HS extract as

antihypertensive agent with elucidation of different mechanisms for such effect. Administration of HS extract to anaesthetized rats caused a considerable decrease in the recorded blood pressure involving both systolic and diastolic pressures. The recorded hypotensive effect was evident despite the prior conditioning of treated animals with different pharmacological agents which were applied to locate the site of HS extract. Atropine did not block the HS action indicating that the cholinergic pathway is not involved in the dynamics of HS. This data is not in accordance with Adegunloye *et al.,* (1996) who found that cholinergic blockade attenuated the hypotensive response to HS. Again, the recorded data is not parallel with Ali *et al.,* (1991) and Bako *et al.,* (2010) who found that the hypotensive effect of HS was blocked by atropine in normotensive cats. The difference in our results with those of other authors may be attributed to the methodological, environmental and species differences. On the other hand, our data may be supported with Adegunloye *et al.,* (1996) who reported that the sectioning of the left and right vagi (cholinergic nerves) did not have a significant effect on the fall of HS-induced fall in the mean blood pressure of the rat. Our data showed that the response of HS was also resistant to prior application of hexamethonium excluding the site of HS action to be autonomic ganglia. Also, the response is not involving adrenergic intervention as beta and alpha adrenoceptor blockers, propranolol and phentolamine, repectively, failed to block the HS response. The in vitro study gave a prominent mechanism of the hypotensive effect of HS that is vasodilation. HS extract relaxed the nor-epinephrine pre-contracted aortic preparations. The relaxant effect was marked and concentration-dependent. Inhibition of the vascular tone in isolated thoracic aorta has been reported previously (Adegunloye *et al.,* 1996; Onyenekwe *et al.,* 1999; Ajay *et al.,* 2007). Nevertheless, the exact mechanism of action of such inhibition remained a controversial matter. Our data proved that the vasorelaxant effect of HS was evident in spite of the pre-treatment of the aortic tissue under investigation with L-NAME, a nitric oxide synthase inhibitor, and nifedipine, a Ca++ channel blocker indicating that HS-induced vasodilation was not due to either nitric oxide-cGMP pathway or inhibiting Ca++ influx pathway as reported by Ajay *et al.,* (2007). The vasorelaxant effect was recorded in all preparations either with intact or denuded endothelia in contrast to what was recorded by Ajay *et al.,* (2007). Such difference may be attributed to methodological and environmental variations. In the present study, glibenclamide, an ATP-sensitive K+ channel blocker failed to block the HS-induced vasorelaxation indicating that the involvement of vascular K+ channels is unlikely. A blocker of K+ channel may result in muscle contraction produced by membrane depolarization. Moreover, involvement of prostacycline in the recorded vascular relaxation seems also unlikely as the pre-treatment of the aortic preparations under investigation in this experiment with indomethasin, a cyclo-oxygenase inhibitor, did not cause any significant changes in the response to HS extract. Taken together, our data may indicate that HS extract may cause vascular relaxation by a direct mechanism on the vascular smooth muscle cells.

The biochemical study may add additional dynamics of the action of the HS as antihypertensive and as antihyperlipidemic as well. In the present study, oral administration of HS extract resulted in decreased serum levels of two leading hypertensive mediators, namely, ACE and Na+. These may lead to decreased amount of activated angiotensin that is a main pressor agent and Na+ that is a hypervolemic ion. Our electrolyte data are partially not in accordance with Mohagheghi *et al.,* (2011) who reported that HS cause no significant changes in serum electrolytes in hypertensive patients in a random clinical trial.

High lipid parameters that usually results in vascular dysfunction and consequently hypertension were also improved to a great extent with HS treatment. The hypolipidemic effect of HS recorded in the present study is in accordance with El-Saadany (1991) who reported that treatment of hypercholesterolemic rats with HS extract for nine weeks progressively lowered various lipid fractions in plasma and tissues. Additional support may come from data of Chen *et al.,* (2003) in rabbits that were fed on cholesterol for 10 weeks, where administration of HS extract to them reduced tri-acylglycerols, cholesterol and low density

lipoproteins in their serum and mitigated aortic atherosclerosis in them. Generally, the recorded various yet parallel effects of HS may be attributed to the various ingredients identified in its different extracts including β-carotene, anisaldehyde, glycinebetaine, trigonelline, anthocyanins, cyaniding-3-rutinoside, delphinidin and delphinidin-3-glucoxyloside (Chen *et al.,* 1993; Meiers *et al.,* 2001; Ali *et al.,* 2005).

Conclusion

It could be concluded that HS extract is a very potential source for development of a potent and cost-effective antihypertensive agent with different but parallel mechanistic pathways including direct vasorelaxation, ACE inhibition, saluresis and hypolipidemia.

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