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Full-Length Research Article

Anti-Hypertensive Effects of Anthocyanins from Hibiscus sabdariffa Calyx on the Renin-Angiotensin-Aldosterone System in Wistar Rats

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Summary: Hibiscus sabdariffa (HS) has gained attention as an anti-hypertensive agent. In the present study, we hypothesized that anthocyanins from HS may attenuate salt-induced hypertension in rats by suppressing the components of renin-angiotensin-aldosterone system (RAAS). Hypertension was induced in the rats by adding 8% NaCl to their constituted diet for six weeks. Wistar rats (n=5 each) were randomly divided into seven groups. Group 1 was the normotensive control group and was fed with normal rat chew and water ad libitum; groups 2 and 3 served as hypertensive control (negative untreated and positive treated with captopril 30mg/kg respectively); groups 4, 5, and 6 served as treatment groups and were administered with graded doses of anthocyanins(50, 100, 200mg/kg respectively) while group 7 received both 100mg anthocyanins and 30mg captopril per day for 4 weeks. Using HPLC, anthocyanins were isolated from HS calyx following standard protocol. Anthocyanins significantly ($p<0.05$) reduced blood pressure and heart rate in hypertensive rats in a dose-dependent manner. The blood pressure reduction by anthocyanins was associated with a reduction in serum ACE and plasma aldosterone in the hypertensive rats. The effects of anthocyanins on blood pressure and biomarkers of RAAS were similar to those of captopril, a reference anti-hypertensive drug. The results suggest that anthocyanins exerts a significant anti-hypertensive potency on rats, probably mediated by the reduction in components of the RAAS.

Keywords*: hypertension, anthocyanins, renin, aldosterone, captopril, rats*

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INTRODUCTION

Hypertension has been identified as a major risk factor for the emergence of cardiovascular disease and is associated with substantial global morbidity and mortality (Gou *et al*, 2011; Okubadejo *et al*, 2019). In Nigeria, the prevalence of hypertension, dependent on the location, is said to range between 28.9% and 46.6%, with a high prevalence of poor control observed among her population (Adeloye *et al*, 2015; Paul *et al*, 2021). In addition to conventional anti-hypertensive drugs, there are several herbal formulations with documented anti-hypertensive potency, and the use of these agents has increased among patients of all socioeconomic levels (Shinta *et al*, 2019). However, there is a general lack of evidence regarding the mechanisms of action and potential interactions of these herbal preparations with routine anti-hypertensive drug therapy (Ajay *et al*, 2007). The anti-hypertensive efficacy of HS extract has been established in various studies (Nwachukwu *et al*, 2015; Abubakar *et al*, 2015).

Anthocyanins constitute one of the components in HS which is associated with anti-hypertensive actions (Ojeda *et al*, 2010). However, there is a paucity of information on the mechanisms of action of anthocyanins in human and animal models of hypertension. The therapeutic manipulation of the RAAS is a known treatment strategy for hypertension (Crowley and Coffman, 2012; Fountain and Lipton, 2019). There is evidence that agents that suppress RAAS have vasopressor and diuretic effects that improve the prognosis in hypertensive patients (Munoz-Durango *et al*, 2016). In available reports, anti-hypertensive activities of crude extracts of HS (Mojiminiyi *et al*, 2012; Abubakar *et al*, 2015) and total anthocyanins mixtures (Cortez *et al*, 2017) were described. The isolation of pure cyanidin-3-glycoside (C3G) as the major bioavailable form of anthocyanins, and the investigation of its anti-hypertensive potency has not been explored considerably. More so, animal studies and human trials have correlated a reduction in blood pressure parameters with the suppression of components of RAAS (Nwachukwu *et al*, 2015; Poulsen *et al*, 2019). Despite this, the mechanisms of action of RAAS that mediate the reduction in blood pressure are yet to be clearly proposed. Isolating anthocyanins and hypothesizing their mechanisms of action on RAAS may be judicious in the management of hypertension. Hence, the present study examined the anti-hypertensive potentials of anthocyanins from HS by evaluating their actions on the various components of RAAS.

MATERIALS AND METHODS

Drugs, Chemical and Reagents: Captopril manufactured by Globela Pharma PVT. Ltd, India and Sodium pentobarbital from the marketers in Nigeria, Macjames Eco-

friendly Products, Nigeria, were acquired for the experiment. Sodium chloride, methanol and distilled water were gotten from the manufacturers – Sigma-Aldrich Chem. Company, St. Louis, USA. Anthocyanins were obtained locally from HS Calyx.

Preparation of Drugs: Captopril was reconstituted in distilled water to give the required dose of 30mg/kg/2ml body weight and was administered orally. Sodium pentobarbital (Nembutal) was used as anaesthesia at a dose of 100mg/kg b.wt given intraperitoneally. Sodium chloride was reconstituted as 8% NaCl and mixed with rat chow. Methanol was reconstituted as 80% methanol and used to extract the constituents of HS calyx. Anthocyanins extracted from HS calyx were used at graded doses of 50mg, 100mg and 200mg/kg body weight.

Experimental Animals: Thirty-five adult male Wistar rats, each weighing between 260g to 300g, were obtained from a local breeder in Akure, Nigeria. They were maintained under standard laboratory conditions at Biosolution Tech. Centre, Akure, fed with commercially formulated chow (Bendel Feeds and Flour Mills, Ewu, Edo state) and had tap water *ad libitum*. Excess feeds and water were removed and replaced daily. After two weeks of acclimatization, the animals were randomly divided into seven groups before the commencement of the experiment.

Plant Collection and Authentication: The fresh calyces of HS were collected from a vegetable garden in Akure, Nigeria. The plant specimen was identified and authenticated by Prof. Lawrence Okoror in the herbarium unit of Biosolution Centre, Akure. A specimen (AAh/207) was deposited in the herbarium.

Preparation of Methanol Extract of HS Calyx: One thousand grams (1000 g) of pulverized HS calyx was cold macerated in 2.5L of 80% methanol for 48hrs. The extract was filtered through filter paper (Whatman Int. Ltd, England). The filtrate was dried using a rotary evaporator (BUCHI Vacuum Controller, V-800) at 40^{o}C under a reduced pressure for 3hrs. The resulting residue (13.5% w/w recovery) was kept under 4^{o}C for the isolation of anthocyanins.

Isolation of Anthocyanins: Anthocyanins were isolated from the resulting residue of HS calyx using standard protocol. Extraction and purification of total anthocyanins were done according to the method described by Ologundudu *et al* (2010). The elution of the extract was done using sepabeads SP-207 resin column (Mitsubishi Chem. Japan). Then the eluate was collected with 60% ethanol and dried by a rotary evaporator at 40^{o}C. Quantification of anthocyanins content in the purified extract was done by the pH differential method described by Lietal (2012).

$$\text{Anthocyanins content (mg/g)} = A \times mw \times df \times v \times 1000/\varepsilon \times L \times wt$$

where:

A = absorbance value; mw = molecular weight of C-3-G, df = dilution factor; v = final volume; ε = absorptive index of C-3-G; L = path length; wt = weight of extract.

The method described by Yang *et al* (2019) was used to determine the content of C-3-G form of anthocyanins using HPLC. The peak areas of anthocyanins from HS were compared with the chromatogram of standard anthocyanins.

Experimental Design and Induction of Hypertension: The rats were divided into seven groups of five rats each according to the method described by Abubakar *et al* (2015)and Balogun *et al* (2016). Group 1 served as normotensive control. Groups 2 and 3 served as hypertensive control (negative untreated and positive treated with captopril 30mg/kg respectively). Groups 4,5 and 6 served as treatment groups and were administered with graded doses of anthocyanins (50, 100 and 200mg/kg respectively). Group 7 received 100 mg/kg anthocyanins and 30mg/kg captopril. Administration of anthocyanins commenced at the end of six weeks of salt loading and lasted for 4weeks between 8am and 9am. All drugs were administered daily by oral gavage.

Hypertension was induced by adding 8% sodium chloride to the feed of the rats daily for 6weeks following the method described by Mojiminiyi *et al* (2012) and also Abubakar *et al* (2015). Weekly systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial blood pressure (MABP) and heart rate (HR) were measured and recorded. Rats with SBP and DBP above 140 and 100mmhg respectively after three consistent readings were considered hypertensive and recruited for the study.

Determination of blood pressure parameters: An automated tail-cuff blood pressure meter, the CODA 11^{TM} NIBP recording system (Kent Scientific Corporation, USA) was used to record blood pressure and heart rate in rats. Rats were placed in restraining holders with a nose cone to calm the animals. The restrainers were placed on a heating pad (32$\pm$2^{o}C) to warm the rat's tail and maintain blood flow to the tail. The rats were placed in the restrainers for at least 5mins before determining their blood pressure. An average of three consistent readings was taken for each rat.

Protocol for serum collection and biochemical assays: The rats were fasted for 12hrs and anaesthetized with sodium pentobarbital (100mg/kg b.wt, i.p) after the last measurement of blood pressure. Subsequently, 2.5ml of fasting blood samples were collected through the cardiac puncture into labeled sterile plain tubes. The samples were allowed to stand for 1hr at room temperature and then centrifuged at 3000rpm for 10mins. The serum was collected with a Pasteur pipette into sample bottles and stored under 4^{o}C until required. All analyses were completed within 24hrs of sample collection

Evaluation of serum renin , ACE and aldosterone: Renin concentration and ACE levels were estimated in the serum using assay kits according to the instructions of the manufacturer (Angiotenin IRIA kits, USA). Aldosterone was estimated in serum using Aldoct A k-2, diasorin, MN as described by the manufacturer.

Ethical Clearance: The procedures and techniques used in the study were in accordance with accepted principles for laboratory animal use and care by the United States National Institute of Health (US NIH Publication, 2011). Ethical

approval was obtained from Research Ethics Committee, College of Medicine, University of Nigeria, Enugu Campus. (Ref. No NHREC/05/01/2508B-FWA00002458-IRB00002323).

Statistical analysis

The values obtained were presented as mean ± SEM. The data were then analyzed using one way ANOVA followed by LSD posthoc test. Values of $p < 0.05$ were considered to be statistically significant.

RESULTS

Total anthocyanins and cyaniding-3-glycoside (C-3-G) contents (in mg/g) and their percentage yield in H. sabdariffa calyx: From each gram of H. Sabdariffa calyx,9.71mg total anthocyanins were isolated – A yield of 0.97%; while 4.43mg C-3-G were isolated per gram of H. Sabdariffa calyx – a yield of 0.44% w/w.

Changes in blood pressure and heart rate in all the groups in response to the administration of anthocyanins and captopril: Treatment with anthocyanins for 4 weeks as presented in tables 1-3, significantly reduced blood pressure parameters in hypertensive rats when compared with their respective baseline values. The mean values of SBP, DBP, MABP and HR in all the treatment groups were significantly reduced when compared with their corresponding baseline values. As seen in table 1, co-administration of anthocyanins and captopril caused maximum reduction in blood pressure and restored BP to normotensive values ($p < 0.001$) especially when compared to the control group.

The mean values of SBP, DBP, MABP and HR of hypertensive rats after 4 weeks of treatment with anthocyanins were significantly reduced when compared with the hypertensive untreated group.

Changes in the serum concentration of components of RAAS in all the groups in response to the administration of anthocyanins and captopril

As presented in tables 4-6, treatment with anthocyanins for four weeks significantly reduced serum level of some components of RAAS when compared with their respective baseline values. The mean values of serum ACE and aldosterone in all the treatment groups were significantly reduced (* is $p < 0.05$, **is $p < 0.01$, *** is $p < 0.001$) when compared with their corresponding baseline values. The tables also show that the mean values of serum ACE and aldosterone after 4weeks of anthocyanins treatment were significantly reduced ($p < 0.05$) when compared with the hypertensive untreated group

Table 1:
Mean Systolic and Diastolic Blood Pressure (SBP and DBP) in mmHg before and after Administration of Anthocyanins

GROUP	SBP Before	SBP After	DBP Before	DBP After
1. Norm	122.42 ± 0.96	124.18 ±162	78.30±1.97	82.08±1.26
2. Hyp-Untr.	195.28±1.73	194.22 ±1.90	139.02 ± 1.13	138.40±0.01
3. Hyp-Cap.	192.50 ± 2.00	135.80 ± 1.64***	139.38 ±1.38	87.58 ± 1.17***
4. Hyp-Ant. 50	194.54 ± 3.19	180.30 ± 1.59*	136.38± 2.11	144.56 ± 1.48*
5. Hyp-Ant. 100	195.60 ± 1.90	156.14 ± 2.26**	139.40 ± 053	100.80 ± 1.31**
6. Hyp-Ant. 200	196.08 ± 1.58	141.04 ± 2.46***	139.48 ± 0.50	99.84 ± 0.28**
7. Hyp-Ant+Cap	192.64 ± 1.97	124.12 ± 1.51***	140.22 ± 0.34	79.46 ± 0.92***

*All values are expressed as Mean±SEM(n=5 in each group); *is p<0.05, ** is p<0.01, *** is p<0.001 vs corresponding baseline values*

Table 2:
Mean arterial blood pressure (MABP) in mmHg before and after administration of Anthocyanins

GROUP	BASELINE (Before)	4TH WEEK (After)
Norm	86.72±0.84	84.64±2.99
Hyp-Untr.	153.30 ± 1.56	154.96 ± 2.15
Hyp-Cap.	151.10±0.97	97.46±1.47**
Hyp-Ant. 50	152.14 ± 1.20	130.44 ± 3.72*
Hyp-Ant. 100	150.84±0.91	126.22 ± 1.04**
Hyp-Ant. 200	150.30 ±1.41	111.22 ± 1.78**
Hyp-Ant+Cap	150.48 ± 1.21	87.24 ± 3.05***

All values are expressed as Mean±SEM(n=5 in each group)
is p<0.05, ** is p<0.01, * is p<0.001 vs corresponding baseline values*

Table 3:
Mean Heart Rate (HR) in beats/minute before and after Administration of Anthocyanins

GROUP	BASELINE (Before)	4TH WEEK (After)
Norm	364.60±3.57	362.18 ± 3.76
Hyp-Untr.	479.22±2.48	476.52±2.43
Hyp-Cap.	475.10±1.69	356.94± 1.82***
Hyp-Ant. 50	472.80±3.87	381.64 ±1.08***
Hyp-Ant. 100	476.50±1.79	379.86±0.29***
Hyp-Ant. 200	477.80±2.13	380.32±0.20***
Hyp-Ant+Cap	477.50±2.48	372.94±2.10***

All values are expressed as Mean±SEM (n=5 in each group)
is p<0.05, ** is p<0.01, * is p<0.001 vs corresponding baseline values*

Table 4:
Mean serum level of renin before and after administration of anthocyanins (ng/mL)

GROUP	BASELINE (Before)	4TH WEEK (After)
Norm	2.06±0.07	2.10±0.05
Hyp-Untr.	2.34±0.07	2.34±0.07
Hyp-Cap.	2.12±0.10	2.38±0.10
Hyp-Ant. 50	1.92±0.04	2.32±0.10
Hyp-Ant. 100	1.96±0.08	2.24±0.09
Hyp-Ant. 200	1.94±0.07	2.20±0.08
Hyp-Ant+Cap	2.02±0.04	2.38±0.07

All values are expressed as Mean±SEM(n=5 in each group)
is p<0.05, ** is p<0.01, * is p<0.001 vs corresponding baseline values*

Table 5:
Mean Serum Concentration of ACE before and after Administration of Anthocyanins (pg/mol)

GROUP	BASELINE (Before)	4TH WEEK (After)
Norm	1.24±0.07	1.24±0.08
Hyp-Untr.	3.46±0.02	3.48±0.02
Hyp-Cap.	3.20±0.09	1.80±0.06***
Hyp-Ant. 50	3.10±0.04	2.48±0.05*
Hyp-Ant. 100	3.02±0.02	2.28±0.02**
Hyp-Ant. 200	3.14±0.07	1.80±0.06***
Hyp-Ant+Cap	3.12±0.04	1.70±0.06***

All values are expressed as Mean±SEM(n=5 in each group)
is p<0.05, ** is p<0.01, * is p<0.001 vs corresponding baseline values*

Table 6:
Mean Serum Concentration of Aldosterone before and after Administration of Anthocyanins (mmol/l)

GROUP	BASELINE (Before)	4TH WEEK (After)
Norm	1.80±0.06	1.86±0.04
Hyp-Untr.	4.60±0.03	4.58±0.04
Hyp-Cap.	4.64±0.02	4.42±0.13
Hyp-Ant. 50	4.60±0.03	2.82±0.02**
Hyp-Ant. 100	4.56±0.02	2.42±0.02***
Hyp-Ant. 200	4.66±0.02	2.44±0.07***
Hyp-Ant+Cap	4.52±0.02	2.38±0.02***

All values are expressed as Mean±SEM(n=5 in each group)
is p<0.05, ** is p<0.01, * is p<0.001 vs corresponding baseline values*

DISCUSSION

Evidence abounds that natural flavonoid from plants, including anthocyanins are associated with improved cardiovascular risk profile (Lelong *et al* 2015; Orororo *et al*, 2018). In our study, using HPLC, 4.43mg cyanidin-3-glycoside (C3G) of anthocyanins were isolated per gram of HS calyx, with a yield of 0.44% w/w. Li *et al*(2012) and Cortez *et al* (2015) have reported that C3G is the major bioavailable form of anthocyanins and that most of the biological effects of anthocyanins have been studied using C3G. In the present study, anti-hypertensive effects of anthocyanins from HS calyx on the RAAS were investigated using the salt-induced hypertensive model in rats. The result showed that the anthocyanins are effective as anti-hypertensive agents by significantly lowering blood pressure parameters and heart rate in hypertensive rats.

The anti-hypertensive potency of anthocyanins in the present study was comparable to captopril, a known ACE inhibitor. Previous studies have reported that HS possesses an ACE inhibitory effect (Ojeda *et al* 2010; Nwachukwu *et al*, 2015). Anthocyanins have been identified as one of the constituents responsible for the anti-hypertensive activities of HS Calyx (Herrera-Arellano *et al*, 2004). We found that anthocyanins at high doses and captopril, a standard anti-hypertensive drug, exert a similar anti-hypertensive action on blood pressure parameters of Wistar rats. Hence, the results in our present study suggest that anthocyanins from HS may be administered alone as an anti-hypertensive agent.

In the present study, co-administration of anthocyanins and captopril caused maximum reduction in blood pressure and restored BP to normotensive values. Hence, co-treatment of anthocyanins and captopril has a synergistic effect and may augment the anti-hypertensive potency of the primary drug. However, earlier workers proposed that co-administration of HS extract and captopril did not induce a further reduction in blood pressure compared to captopril alone (Shinta *et al*, 2019). The discrepancy in the action of anthocyanins and crude HS extract in combination with captopril may be due to other components in HS like quercetin which could lower the amount of captopril absorbed into the blood circulation (Barrenetrxe *et al*, 2010). RAAS is a known physiologic regulator of blood pressure and it has been speculated that a reduction in blood pressure is associated with a down-regulation of components of the RAAS (Poulsen and Fenton, 2019). The attenuation of the three basic components of RAAS has been reported to be an important mechanism of action for blood pressure reduction (Munoz-Durango *et al*, 2016). In the present study, anthocyanins at high doses, similar to captopril and a combination of anthocyanins with captopril, significantly reduced serum ACE in hypertensive rats. This finding is consistent with the known action of ACE inhibitors (Shinta *et al*, 2019) and HS extract (Ojeda *et al*, 2010). The observed reduced serum ACE by anthocyanins possibly reduced the conversion of Angiotensin I to Angiotensin II and consequently reduced the pressor response mediated by angiotensin II, leading to a reduction in BP (Nwachukwu *et al*, 2015). The ACE inhibitory effect of anthocyanins in this study was dose-dependent. The highest effect of anthocyanins on serum ACE was obtained in a combination of anthocyanins and captopril. The findings agree with Shinta *et al* 2019 who reported that HS extract used as a supplement with captopril may cause a synergistic anti-hypertensive action. In the current study, anthocyanins significantly reduced plasma aldosterone in hypertensive rats. Previous reports suggested that aldosterone levels decrease with ACE inhibitors (Nwachukwu *et al*, 2015). However, others have reported higher levels of aldosterone in hypertensive rats treated with ACE inhibitors (Sato *et al*, 2003) probably due to the escape of aldosterone from ACE inhibition (Sato *et al*, 2003). The observed reduced serum aldosterone by anthocyanins possibly reduced the reabsorption of fluid and electrolytes leading to a reduction in blood volume and blood pressure (Poulsen *et al*, 2019).

We found that anthocyanins, similar to captopril and a combination of anthocyanins with captopril did not significantly reduce plasma renin in hypertensive rats. However, studies have shown that HS caused a significant reduction in plasma renin levels of hypertensive rats (Joven *et al*, 2014). The lack of a significant reduction in plasma renin in the present study may be due to compensatory renin release resulting from reduced ACE and aldosterone (Ojeda *et al*, 2010). Hence, the present results suggest that renin levels in hypertensive rats treated with anthocyanins are not correlated with blood pressure reduction.

In conclusion, anthocyanins from *H. sabdariffa* calyx similar to captopril, exert a significant blood pressure-lowering effect in salt-induced hypertensive rats. The blood pressure reduction may be mediated by the reduction in serum ACE and plasma aldosterone. Thus, anthocyanins may be an effective anti-hypertensive agent either alone or in combination with a known anti-hypertensive drug.

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